ADRENAL CORTEX

Transactions of the Fifth Conference
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2d Row *Peggy Kubie Konrad Bloch Walter Bauer Choh Hao Li* Dwight J *Ingle* Edwin B Astwood Abraham White Ralph I Dorfman Jerome W Conn Ruth E Rue T F Gallagher H L Mason

TABLE OF CONTENTS

Fifth Conference on Adrenal Cortex

THE JOSIAH MACY, JR FOUNDATION CONFERENCE PROGRAM

When I was on a destroyer out at Bikini in 1946 I was fascinated listening to our radio operator as he tested communication equipment He would ask another ship through his radio "How do you hear me?" and the answer often would come back I hear you Nine Nine Nine That meant that everything was satisfactory Of the three nines one was for intensity one for clarity and one for meaning

The Josiah Macy Jr Foundation has organized and devoted a large portion of its resources to the support of its Conference Program because the officers are cognizant of the fact that there is considerable obstruction to communication and mutual understanding across the disciplines and specialties and that this in fact is one of the major factors delaying scientific advance We feel that there are psychological as well as semantic factors contributing to the difficulty of communication people even in arguments with one another are too much inclined to make statements *at* rather than to communicate *with* others I think that we are inclined to forget though that the real question is are these words and statements those which are likely to convey to the listener the whole or even a small part of what I would like to express

I have a feeling that we should be very much concerned with the other fellow's receiving set and not only with our own transmitter If the other person doesn't seem to understand us it may not be enough merely to increase the power of our transmission we must try to find the obstruction in his receiving set, and see what kind of filters and resistors he uses So if we call out to the interprofessional No-Man's Land "How do you hear me?" and the reply comes back "I hear you Nine Nine Nine" we have the beginning of communication What we try to do in these conferences conducted by the Foundation is to set the stage for meaningful communication

With the accelerating rate at which new knowledge is accumulating and with the increasing recognition that nature is of one piece it becomes evident that the continued isolation of the several branches of science from one another is a serious obstacle to scientific progress Nowhere in science is the need for combined opera

tions more evident than in medicine Today to be effective medical research and practice must embrace data from all the disciplines including nuclear physics at one end of the spectrum and cultural anthropology at the other for advances in one field are frequently dependent upon knowledge derived from quite another discipline

Although the fertility of the multidiscipline approach is thus recognized universities and scientific societies and journals which are usually restricted to one small area of a field in their coverage have not yet made adequate provision for channels of interdisciplinary communication We do not wish to compete with the formal scientific meetings or with the scientific journals which have established patterns and formats for the presentation of material Our purpose at the meetings is to keep an informal atmosphere and to encourage the exchange of methods research plans concepts and difficulties which cannot be done if there is formal speech making

The Foundation has endeavored to meet the need for interdisciplinary communication by bringing together for a series of two and a half day annual conferences a small group of investigators representing in so far as possible all the branches of science related to a chosen problem Participants in these informal conferences over a five year period develop a feeling of friendship trust and mutual respect which in turn promotes communication cross fertilization of ideas and cooperation The success of such an endeavor however is dependent upon full participation of all members in the discussion Accordingly attendance at any conference is limited to twenty five

Under the guidance of Dr Willard C Rappleye President of the Foundation since 1942 the Conference Program has been gradually expanded and enlarged until during 1953 it included twelve different groups which meet annually to discuss a wide variety of problems in the field of medicine and the closely related disciplines Our plan is to discontinue the meetings of each group at the end of five years

In order to share with a wider group of investigators and students the essential quality of these conferences and to give others an insight into the functions of the scientific mind the informal nature and tempo of the discussions as far as possible are preserved in the published transactions

FRANK FREMONT SMITH M D
Medical Director

THE SALT AND WATER FACTOR OF THE ADRENAL CORTEX*

H L MASON
Department of Biochemistry
Mayo Clinic

YOU ARE ALL I think familiar with the works of Tait Simpson and Grundy (1) and Woodford (2) in England More recently Reichstein and his associates (3) have announced the isolation of what has tentatively been called "electrocortin Dr Reichstein has indicated to us that electrocortin is not a satisfactory name but feels that a better name should at least await the determination of the structure

We were a little behind Reichstein in announcing the crystallization of our compound which appears to be the same one that he and his associates have isolated A lack of materials on both sides has prevented a comparison of the two preparations Briefly our preparation involved column chromatography first on silica gel columns We had difficulty in obtaining clean separations of our active material from cortisone so we turned to acetylation Perhaps that was a hazardous procedure inasmuch as Grundy *et al* (2) stated that acetylation of their material destroyed its activity Of course when one speaks of activity one should indicate the level at which it has been tested we found a considerable amount after acetylation of a test portion of the material Therefore we decided to acetylate it

Figure 1 summarizes one of our first columns The fractions containing compounds A and B are indicated and our activity is bracketed between the two vertical lines which include compound E In accordance with the findings of Grundy *et al* (2) we also found that the activity traveled with much the same mobility as cortisone (Figure 2) After acetylation however we found that the sodium retaining activity was much more mobile than cortisone acetate and that we could achieve a clean separation of the latter The zone containing the sodium retaining activity also contained a

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*Associates in this work were Drs V R Mattox and A Albert of the Mayo Clinic Rochester Minnesota

Mg α β-unsaturated ketone as cortisone

7
6
5
4
3
2
1

A
B
E

5 10 15 20 25

Fraction number

Solvent system Propylene Glycol–Tol ene

FIGURE 1 Fractionation of beef adrenal extract by column chromatography

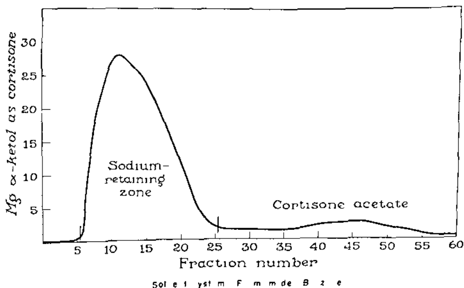


FIGURE 2 Fractionation of cortisone zone by column chromatography after acetylation

number of other substances some of which were crystalline but the nature of which we do not know

The acetate in our assay appeared to have about the same activity as desoxycorticosterone acetate (DCA) which of course was not very encouraging but in view of the statement of Grundy *et al* (2) we thought we should hydrolyze it (Figure 3) The acetate

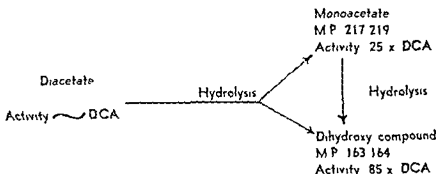


Figure 3 Enzymic hydrolysis of diacetate of sodium retaining compound

which we have designated at least tentatively as diacetate was treated with citrus acetylesterase After paper chromatography of the product of hydrolysis we found there were two substances one of which we called monoacetate and the other the dihydroxy compound." Those designations are tentative but serve to identify the particular fractions

The monoacetate crystallized first it had an activity of approximately 25 times that of desoxycorticosterone acetate which was encouraging in view of the low activity of the diacetate Eventually we crystallized the dihydroxy compound and found it had an activity of 85 or perhaps 100 times that of desoxycorticosterone acetate which is comparable with the compound described by Simpson Tait Reichstein *et al* (3) Their compound has been described as having an activity of from 50 to 100 times that of DCA

Figure 4 is a paper chromatogram showing the mobilities of the three compounds just described the diacetate the monoacetate and the free compound

Figure 5 is a comparison of the free compound and the mono acetate with cortisone and cortisone acetate You will note that the mobilities of cortisone and of the free compound are very similar whereas the mobilities of the acetates are very different As to the

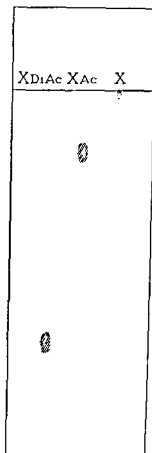


FIGURE 4 Paper chromatography of sodium retaining substance and its mono- and diacetate

chemistry of the compound by the use of ultraviolet and infrared spectroscopy we found that there was an alpha beta unsaturated ketone group which was also identified by Reichstein and of course it is the easiest thing to find In conformity with the known adrenal hormones the obvious thing is to assign the ketone group to the 3 position and the double bond to the 4 5 position in conjugation with it Also by infrared spectroscopy we have evidence of an alpha ketol side chain that is the spectrum of the acetate is in agreement with the spectra of the 21 acetates of the known steroids with alpha ketol side chains The substance reduces

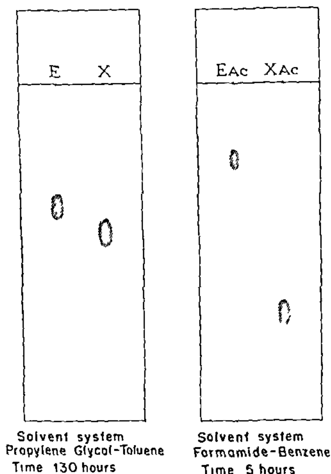


FIGURE 5 Paper chromatography of free hormones and acetylated derivatives

tetrazolium promptly Thus unless this is an entirely unorthodox steroid we have placed three oxygen atoms Lacking an elementary analysis we do not actually know whether it has four or five oxygen atoms we have evidence of only four We have been unable to identify the presence of another ketone group but cannot be sure that it is not there

However we think that another hydroxyl group in addition to the three already mentioned would account for the mobility in chromatography Where it is placed remains to be seen but we think we have rather definitely eliminated position 16 The compound does not give a Porter Silber reaction and we have been

informed by Dr Gordon Farrell of Western Reserve University that 16α hydroxy 11 desoxycorticosterone does give the Porter Silber reaction A recent communication by Hirschmann, Hirschmann and Farrell (4) stated that the latter compound does not have the high salt retaining activity of electrocortin Thus on chemical and biological grounds one may I think eliminate the possibility of its being a 16α hydroxy 11 desoxy compound The negative Porter Silber test also pretty well rules out the presence of the 17 hydroxyl group as does the absence of a hydroxyl band in the infrared spectrum of what we have called the diacetate

We have made the 3 dinitrophenylhydrazone of the diacetate and the 3 dinitrophenylhydrazones of 6 alpha and 6 beta hydroxy 11 desoxycorticosterone When the latter two were heated with perchloric acid and acetic acid the acetic acid was split out, and the absorption maxima shifted from 10 to 15 mμ toward longer wave lengths In the case of the unknown compound there was no shift in the spectrum under such treatment Thus we believe there is little possibility that a 6 hydroxl group is present We think perhaps this experiment with the dinitrophenylhydrazones would make the presence of a hydroxyl or acetoxyl group unlikely at positions 1 and 2 We have no model compounds so we cannot be entirely certain of that It probably eliminates position 7 also

Next for consideration is the 11 alpha position A hydroxyl group in that place would give the 11 isomer of corticosterone which has also been tested biologically and has been found to have very little if any of the growth promoting activity associated with sodium retaining activity Because the unplaced hydroxyl group was acetylated under very mild conditions we think the tertiary positions have been ruled out Remaining then are positions 12 and 15 and the two angular methyl groups 18 and 19

I stated that we think one more hydroxyl group in the right position combined with the other structural features of desoxycorticosterone would account for the mobility of this material on paper That conclusion is supported by the fact that 16α hydroxy 11 desoxycorticosterone moves with about the same mobility as cortisone

Under conditions which would form the 21 acetate we have prepared a monoacetate which was different from that obtained by enzymatic hydrolysis of the diacetate When this monoacetate was oxidized with chromic acid the product moved at a rate which would be expected from the conversion of a hydroxyl group to a

ketone group This result furnishes further evidence that we are dealing with a secondary hydroxyl group

The physiological properties of this compound are unknown except that it does have a high degree of activity in causing retention of sodium and excretion of potassium We have necessarily been confined in our physiological studies to an assay method in order to isolate the material

Long Dr Mason has set before us an extremely interesting and provocative topic For many years we have suspected that there might be a substance in the adrenal cortex which has an effect on mineral metabolism It now appears to be a fact Whatever the structure of the compound may prove to be we certainly await with a great deal of interest further analyses of its physiological properties

Kendall I should like to congratulate Dr Mason on a piece of work which is of high order When one considers the modest amount of material at his disposal it is evident that it took a great deal of courage to go forward with only 2 98 mg I think we will all agree that he has uncovered some definite evidence

I believe the most important aspect of the problem now lies in what else the molecule does physiologically in addition to the effect on the metabolism of sodium and potassium and the distribution of water We have associated certain physiological activities with cortisone and hydrocortisone but the effect produced by those two hormones on mineral metabolism is quite inadequate for us to accept them as the only active agents in the gland

Finally we come to the mechanism which regulates the amounts of this substance as well as cortisone and hydrocortisone Apparently the amount of the material is not increased by the administration of ACTH Is the level of sodium regulated by the activity of the gland which in turn is regulated by the level of sodium that is is it like calcium and the parathyroid? The other hormones cortisone and hydrocortisone are under the control of ACTH It would be very interesting to know the entire mechanism for the control of these three hormones that is cortisone hydrocortisone and the sodium factor unless we crossed off cortisone and ascribed the effect on organic metabolism to hydrocortisone That would bring the number of hormones down to two

Long Dr Kendall showed that the amount of this new substance in the adrenal blood is not increased by ACTH

Kendall It has been known for a long time that after hypophysectomy the animal does not go into too much of a decline from

loss of control of the metabolism of sodium Dr R C de Bodo (5) has done some nice work on the effect of hypophysectomy on the adrenal gland The animal does very well with regard to sodium metabolism

Long The situation in the hypophysectomized animal is a relative one I think Gaunt (6) and others have shown it cannot respond to low water and salt in the way a normal animal can it suffers from water intoxication to the same extent as an adrenalectomized animal If this newly isolated substance alone is involved under those conditions one would suspect that there must be an increase Also has it not been shown that in the case of many adrenalectomized humans administration of cortisone or hydrocortisone is all that is required to maintain the mineral as well as the organic metabolism

Kendall I think that varies with the individual In some it is certainly true

Mason Some patients with Addison s disease do better when they receive both cortisone and desoxycorticosterone

Kendall How about the patients who have been adrenalectomized for various reasons? Some I believe get along without desoxycorticosterone Is that right?

Conn Yes some of them do but the majority require a small amount of desoxycorticosterone perhaps a milligram a day

Mason Didn t Dr Charles Huggins of the Ben May Laboratory for Cancer Research University of Chicago treat all his patients with cortisone alone with one or two exceptions?

Conn I think so

Long May I ask the method of assay you employed Dr Mason?

Mason The assay method is not very different from that of Simpson and Tait (7) except that we used the flame photometer for determination of sodium and potassium rather than radio activity It also differs in some other details Rats of about 150 gm weight were adrenalectomized and kept on an ordinary diet with salt water for drinking Twenty four hours before the assay they were taken off the salt water The test substances were injected and the urine collected over a three hour period

Ralli Did you hydrate the rats and then administer the test substance?

Mason No

Ralli You simply withheld salt?

Mason Yes just overnight

Ingle Dr Mason who isolated the 16α hydroxy compound?

Mason Dr Hans Hirschmann and his associates (4) have synthesized 16α hydroxy 11 desoxycorticosterone

Ingle Has only the one isomer been prepared?

Mason That is my understanding

Pincus I think we might eliminate position 12 Dr Dorfman didn t you study 12 keto 11 desoxycorticosterone a number of years ago?

Dorfman Yes I studied the substance prepared by Dr R D H Heard of McGill University Montreal and it was active but con tained from 1 to 20 per cent of desoxycorticosterone That was reported in one of our early papers (8)

Mason Was it 12 hydroxy?

Dorfman Yes I believe so

Pincus Alpha or beta?

Dorfman The designation I think was not specified at that time It would have to be checked

Pincus We had the 12 ketone from Dr Heard and it had about 1/100th the activity of 11 desoxycorticosterone Thus I should think that unless this is a very unusual compound that requires a hydroxyl function for especially high activity position 12 might very well be eliminated by the same type of reasoning as applies to some of the others I think position 15 should also be excluded because as I remember the work of Simpson and Tait (9) the remaining and unknown hydroxyl is a rather strongly bonded hydroxyl according to the infrared spectrum Isn t that correct?

Mason I was not aware that they had an infrared spectrum

Kendall No I do not think they do They did not even sug gest 15

Pincus What does your infrared spectrum show? Isn t it strong? If so position 15 would be unlikely

Mason We actually have not had an infrared spectrum on the compound with the free hydroxyl group in the unknown position

Pincus You have had all the acetate?

Mason All the diacetate Dr Norman Jones took a spectrum for us on the monoacetate which we have assumed has the X position acetylated and the 21 hydroxyl group free We have no evidence for that so I cannot answer the question

White Did I understand you to say that Dr Hirschmann has made the 16 alpha desoxycorticosterone but that so far as you know the 16 beta has not as yet been made?

Mason That is correct

Young Is there any reason to suppose that the 16 beta hydroxyl would give the Porter Silber reaction?

Mason I cannot answer that but I think it probably would

Young You would eliminate 16 beta on that basis?

Mason Tentatively at least I would reason by analogy from the 6 alpha and 6 beta hydroxylated compounds There is a difference in the yield of the Δ^6 compound from the two isomers one dehydrates or loses acetic acid more readily than the other Offhand I do not know any reason why a 16 hydroxyl group should not behave similarly

Pincus The 16 beta would be very labile to acid I should imagine

Mason That would certainly give the Porter Silber reaction We found that compounds with the delta 16 bond gave the Porter Silber reaction so it appears that that reaction involves a dehydration between the 16 and 17 carbon atoms

Bloch If there is a hydroxyl at one of the angular methyl groups one should obtain an aldehyde on oxidation instead of a ketone Moreover if 15 is eliminated there are really no positions left

Mason The analyses were done on just a few micrograms We thought the angular methyl groups were probably eliminated by the oxidation procedure Of course so much of our evidence is tentative that I do not think any chemist would be entirely satisfied with it

Long What is the best estimate of the amount of this corticoid that is present in a kilo of glands? At our conference last year Dr Martha Vogt (10) gave the estimates of Simpson and Taft What is your experience as to the quantity present in a kilo of glands compared to the other steroids?

Mason I have not actually calculated it We obtained an amount similar to that of Reichstein who mentions 500 kilos yielding 22 mg in another place he speaks of 1 100 kilos yielding about the same I think the latter figure is probably correct Thus there would be about 2 mg per 100 kilos

Ingle If this compound is very labile in the fractionation procedures the amount *isolated* would not necessarily reflect the secretory activity of the gland would it?

Mason We have no idea as to what the losses might be during the preparation but of course every effort was made to keep the conditions as mild as possible For that reason we used partition chromatography rather than adsorption chromatography

Young Do you have any positive evidence that the substance is present in the amorphous fraction? Or do you assume that by exclusion because it is not present in other fractions?

Mason Our first experiment was directed toward that very thing We prepared some amorphous fraction as had been done in the past, and although we could not positively identify the active material at least the mobility on paper appeared to be the same as in the material which we had isolated We have not actually compared the two since we do not have any more of the amorphous fraction

Pincus Dr Mason you said that it was difficult to separate this material from compound E by the usual method Is cortisone a constituent of the amorphous fraction?

Mason Cortisone compound F corticosterone and a dozen other things are present

Pincus The point I am trying to get at is this cortisone has been separated from adrenal extract with ease Why do you say that this material is characteristic of the amorphous fraction? Why isn t it one of the things that goes along with cortisone in the usual fractionations for cortisone?

Mason I do not know that I can answer that with any authority We have not tested natural cortisone for this type of activity Have you Dr Kendall?

Kendall Prof W W Swingle and his associates have tested cortisone for its ability to retain sodium It is very weak The sodium factor does not separate with cortisone and the other crystalline material at all

Pincus It is in the mother liquor?

Kendall Yes all of the sodium retaining material is there

White Dr Mason you said did you not that your experience with the dinitrophenylhydrazone behavior indicated that position 6 should be eliminated and you felt that this evidence ruled out positions 1 and 2 and probably 7 as well?

Mason I said "possibly

White Some of us have had experience with bioassays of the 2 hydroxy compounds and it can probably be said that the 2 alpha hydroxy compounds are quite inactive I do not know whether anyone here has had any experience with the 2 beta hydroxy compounds

Another point from the standpoint of chemistry how would you interpret the structure in terms of the significant degree of destruction of activity on acetylation?

Mason That has puzzled me a great deal and I have no constructive suggestion to make about that It was surprising to find that uncovering just the 21 alcohol group increased the activity about 25 to 30 times

White How is this material administered for bioassay?

Mason It is given subcutaneously in a 1 1 mixture of propylene glycol and water

Ralli Does the effect occur because the increased rate of urine output increases the excretion of potassium?

Mason It occurs because of the amount of sodium and potassium excreted There is a retention of sodium and a stimulation of potassium excretion Simpson and Tait (7) used the sodium and potassium ratio and we also found that to be a better index than measuring the sodium retention alone

Kendall Do you always determine both sodium and potassium and use the ratio of sodium to potassium?

Mason Yes

Dorfman How about life maintenance? Does it parallel this high activity?

Mason We have not done that but people at the Upjohn and Company laboratories have Dr Ingle do you remember what they said about this effect?

Ingle I cannot remember any ratios but I know that this principle was many times as potent as DCA in the life maintenance test and in supporting the growth of the immature adrenalectomized rat

Kendall It was not as much as desoxycorticosterone?

Mason Many times more

Kendall Not as much as 80 or 100?

Mason No

Young I think I have seen some data recently which suggested that if the urinary sodium potassium ratio is taken as criterion which is what Simpson and Tait did the material is approximately 100 times as active as desoxycorticosterone but in the case of life maintenance it may be 30 times as great If we take sodium retention in the dog it is about 50 times as active

Long Is that about the ratio you found with your old amorphous fraction Dr Kendall?

Kendall There is a figure of 2 gamma per kilo in an old paper of mine (11)

Long Did you use the dog originally maintained on a low potassium high sodium diet?

Kendall No not high sodium I think we should recognize the time factor Dr Mason speaks of three hours If one injection or even two are given in a day the animal must be sustained for the entire 24 hours Thus I would not be surprised if this value of 100 were to shrink to 20 or 30 on a life maintenance or growth basis

Long There is no evidence at all so far as to whether the material has or has not any effect on carbohydrate or protein metabolism?

Mason Not in the case of crystalline material As to the old amorphous fraction I think Dr Ingle could answer the question

Ingle It had a greater effect than desoxycorticosterone but much less than cortisone when assayed in the work test

Long An amorphous fraction might have contained some cortisone

Mason It undoubtedly contains cortisone and also hydrocortisone

Long Do the crystals so far as is known possess any activity other than the effect on water and salt metabolism?

Mason If so it has not been measured as yet

Young Knauff Nielson and Haines (12) say that it has no effect on carbohydrate metabolism but it is rather risky to say that it has no activity without knowing the dose used Do you recall what dose they administered?

Mason I am sure I have heard them say but I do not remember exactly

Pincus They did not state the dose in their paper Is there any evidence of other electrolyte regulating substances in the amorphous fraction?

Mason We have not found appreciable activity in any other fraction but we have not looked very thoroughly We are centering our attention on the fraction that gave us the greatest activity

Long I should like to get back to the lack of effect of ACTH Has any other pituitary hormone been tried? I am thinking particularly of what Dr Selye has suggested that there may be another pituitary factor that controls the secretion of the water and salt regulating hormone Has growth hormone been tried?

Li Growth hormone has been demonstrated to retain sodium and potassium in both normal and adrenalectomized rats (13)

Long That is not quite the same thing as we are talking about here

Conn Dr Long the only other suggestion about a relationship is the effect of ACTH on nephrotic patients who are excreting

large amounts of this material Upon cessation of ACTH this electrolyte active factor disappears from the urine with the diuresis

Long The suggestion is that this condition was stimulated by ACTH?

Conn Yes

Pincus I believe that in the analysis of adrenal vein blood Dr I E Bush (14) found increased amounts of this material after ACTH We found it in perfusates I cannot state unequivocally that it is increased after ACTH administration However I think *it must be because the experiments in which it has been shown to* be present have all been found in ACTH perfused adrenals

Ralli If the material has a water retaining effect one would expect it to increase the reabsorption of water and thus decrease the volume of urine excreted *Did you measure the urine volume?*

Mason No we have not attempted to do that

Pincus If it acts like DCA in the acute test with the rat there should be a definite decrease of urine volume That is one of the characteristics at least of desoxycorticosterone Are the animals given a salt load Dr Mason?

Mason Dr A Albert of the Mayo Clinic has been doing the assays and I cannot speak too authoritatively because the details have been changed from time to time however as I understand it no salt load was given That was done at first but not later

Long However they drink one per cent sodium chloride to maintain them?

Mason Yes

Long That is a fair level of salt intake?

Mason No it is not very much

Long This compound recalls one of the central questions of adrenal physiology which is the apparent separation of the effects on water and salt metabolism of compounds of this type and the effects of cortisone on the organic metabolism Nevertheless I understand that cortisone and hydrocortisone when they are employed in sufficient amounts have a complete effect on both the organic metabolism and inorganic metabolism

Dorfman It is a question of what you mean by complete After all desoxycorticosterone can increase the glycogen level of the fasting animal both in the rat and mouse It has about one per cent of the activity of hydrocortisone

Ralli It might not be due solely to the hydrating effect of DCA on the animal If the general condition of an animal is improved

by the administration of desoxycorticosterone his capacity to absorb from the intestinal tract may also be improved

Dorfman These are fasted animals

Long And also adrenalectomized?

Dorfman Yes

Long I believe it is a different story in the intact animal in that case have you ever seen any glycogen decomposition from desoxy corticosterone?

Ingle Exacerbation of diabetes will occur if 10 mg of desoxy corticosterone per day is given to the partially depancreatized rat

Long Is this in an adrenalectomized animal?

Ingle The adrenals are intact

White I assume the uncertainty about four versus five oxygen atoms is largely due to inadequate amounts of material for elemental analysis However from the standpoint of its polarity the chances are that it is four

Mason Yes we think four is probable

EDITOR'S NOTE Since this discussion took place Dr Reichstein and his associates (15) have reported the following findings

> Chemical degradation of the new crystalline mineralocorticoid provisionally called electrocortin has shown that this compound is 11β 21 dihydroxy 3 20 diketo-4 pregnene 18 al In solution this reacts mainly as the 11 hemiacetal We suggest aldosterone as a definitive name for the compound

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THE METABOLISM OF ADRENAL STEROIDS

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As you all know, the metabolism of adrenocortical hormones is a complex subject. I shall limit my discussion to the metabolism of adrenocortical steroids in man and present some recent speculations on this problem. Furthermore, we shall consider *in vivo* or over-all metabolism. This is an important phase of metabolism, particularly for clinical medicine. If we could relate the endogenously produced adrenocortical steroids to specific metabolites in the urine and if these metabolites could be quantitatively determined, we would then have a rational system for determining the specific status of the adrenal cortex. I shall present for your consideration (*a*) a summary of the known facts about the relationship between the tissue steroids and the metabolites in the urine, (*b*) a generalization of steroid metabolism which leads to a method for the calculation of quantities of steroids endogenously produced, (*c*) an analysis of the meaning of group steroid reactions as applied to urinary extracts, and (*d*) a working hypothesis concerned with the biochemical defects that lead to various clinical types of adrenocortical hyperactivity.

At the moment we have evidence to indicate that a variety of adrenal steroids are secreted into the blood stream. These substances are metabolized by various peripheral tissues and finally appear in the urine. The greatest amount of work has been concerned with the urinary compounds.

I should like to consider now the reduction of Ring A of the steroid nucleus, a story that will lead us to a working hypothesis. All of the neutral steroid hormones that possess biological activity thus far studied contain in Ring A an α,β unsaturated ketone or more specifically Δ^4-3-ketone. One of the characteristic features in metabolism in the human as well as in the animal is the reduction of the ketone group to a secondary alcohol and the saturation of the 4,5 double bond (1).

I should like to present for discussion the thesis that the type of reduction of the Δ^4 double bond is regulated by the constituent

groups on the steroid molecule That is the reduction is oriented either into one or another stereoisomeric form which may be designated as alpha (androstane or allopregnane) or beta (etio cholane or pregnane) In the C_{19} series (androgens) compounds of the 5α configuration are called the androstane group while those of the 5β series are of the etiocholane group In the C_{21} series of steroids which include progesterone and adrenocortical hormones 5α will designate the allopregnane series while 5β will refer to the pregnane compounds

We now *come back to the general thesis that substituent groups* at carbon atoms 11 and 17 influence the way in which the double bond at carbon atoms 4 and 5 are reduced When C_{19} Δ^4 3 ketosteroids with a 17 oxygen function either as a hydroxy or a *ketone group (such as testosterone or Δ^4 androstene 3 17 dione)* are present in the body these compounds are reduced so that the ratio of the 5β to 5α form varies between 0 6 and 3 7 with a mean of about 2 1 If on the other hand we deal with compounds having the same 19 carbon atoms but with an additional oxygen at position 11 such as in adrenosterone the ratio of 5β to 5α stereoisomers is something of the order of 0 2 (Table I)

TABLE I

Metabolism of Δ^4 3 Keto-C_{19} Steroids to Saturated C_{19} Steroids *In Vivo**
(Humans)

Steroid Administered	No of Experiments	Ratio 5β/5α† Mean (Range)
Testosterone	4	1 89 (0 6 3 70)
Δ^4 Androstene 3 17 dione	4	1 32 (1 02 2 23)
Dehydroepiandrosterone	5	1 83 (0 6 4 6)
Adrenosterone	1	0 19

Data of Schiller *et al* (2) Dobriner (3) Gallagher *et al* (4) Dorfman (5) Ungar and Dorfman (unpublished) Mason and Kepler (6) Miller *et al* (7) Savard *et al* (8)

†5β = etiocholane 3α ol 17 one or 11 oxygenated etiocholane 3α ol 17 one

5α = androsterone or 11 oxygenated androsterone

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If we have a Δ^4 3 ketosteroid with a side chain at carbon 17 as in the case of progesterone we find that in metabolism (reduction of Δ^4 group) there is a preponderance of the 5β stereoisomer formed Actually in the experiments in the literature and our own experience the mean ratio of 5β to 5α form is something of the order of 21 1 (Table II) Studies with desoxycorticosterone 11 dehydrocorticosterone cortisone and other C_{21} steroids have yielded Ring A reduced compounds only in the pregnane series (Table II) Some of these steroids contain oxygen functions at 11

TABLE II

Metabolism of Δ^4 3 Keto C_{21} Steroids to Saturated C_{21} Steroids *in Vivo**
(Humans)

Steroid Administered	No of Experiments	Ratio $5\beta/5\alpha$ Mean (Range)
Progesterone	4	21 (7 5 33 0)
Cortisone	2	∞†
Hydrocortisone	1	∞†
21 Desoxycortisone	2	∞†
Desoxycorticosterone	4	∞†
11 Dehydrocorticosterone	1	∞†
Δ^{16} Dehydroprogesterone	1	∞†
11 Ketoprogesterone	1	∞†

Data of Burstein *et al* (9 10 11) Horwitt *et al* (12) Ungar *et al* (13) Dobriner and Lieberman (14) Kyle and Marrian (15) Savard and Dorfman (unpublished) Mason (16)

†No allopregnane derivative thus far isolated

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as in cortisone and corticosterone in addition to the presence of a side chain When both C 11 oxygen and a C 17 side chain are present it appears that the influence of the side chain is more important than the 11 oxygen This is true since the type of metabolites we obtain in the pregnane series are similar to the type found for C_{21} compounds with an 11 oxygen substitution

There is a suggestion that in the metabolism of a compound such as cortisone to 17 ketosteroids two pathways exist That is one pathway (A) where the formation of a dihydro derivative (5β series) would be produced by two hydrogens saturating the double bond at 4 5 which in turn would be converted to a 17 ketosteroid of the 5β form and a second pathway (B) where cortisone would be oxidatively converted to adrenosterone which would be metabolized on both 5β and 5α reduced forms Thus from route (A) we obtain a ratio of 5β to 5α forms of about 21 1 and from route (B) a ratio of 1 5 The total products from routes (A) and (B) have been determined to be of the order of 6 5 1

Table I presents the *in vivo* metabolism of three $C_{19}O_2$ steroids and one $C_{19}O_3$ steroid Specifically testosterone Δ^4 androstene 3 17 dione and dehydroepiandrosterone when administered to the human are metabolized in such a manner that there is a slight preponderance of the 5β reduced form Tables I and II as well as in Table III which is to follow the difference in stereoisomerism at carbon atom 5 is emphasized When $C_{19}O$ compounds are metabolized the end products are androsterone (5α) and etiocholane (5β) We observe in Table I that the mean ratio of the 5β to the 5α isomers was 1 89 and varied from 0 6 to 4 6 In contrast to the 5β to 5α ratio 1 89 for the $C_{19}O_2$ steroids the metabolism or reduction of adrenosterone a $C_{19}O_3$ compound resulted in a preponderance of the 5α form Here the 5β and 5α isomers refer to the 11 oxygenated derivatives but the type of reduction at carbon 5 is similar to that of the $C_{19}O$ compounds In Table I therefore we have suggested that the presence of an oxygen function at 11 influences the manner in which the Δ^{4} group is reduced

Table II deals with the metabolism of the Δ^4 3 keto C_{21} steroids which are reduced to the saturated C_{21} *steroids Here we have* eight different compounds which are known to be reduced in Ring A Using again the same convention of 5β and 5α stereoisomers we find the interesting fact that in all instances the important reduction product quantitatively is the 5β form Actually in the *case of progesterone* for which the greatest number of experiments were reported the preponderance of 5β stereoisomer is indicated by the fact that the mean 5β to 5α ratio is somewhere in the neighborhood of 21 varying from 7 5 to 33 Thus the important fact that this table illustrates is that the presence of a side chain at carbon 17 with or without an oxygen function at 11 orients the reduction of the 4 5 double bond to the β form

White How do you bring into harmony the 11 keto progesterone and progesterone?

Dorfman The only metabolite of 11 progesterone that I know of is 11 ketopregnanediol which is in the pregnane series The point is that in each of these instances a pregnane derivative has been isolated

White With DCA then as compared with progesterone is the nature of C_{21} significant? I am concerned with the fact that you are making a good deal of whether or not there is an 11 oxygen as compared to the nature of the side chain because in DCA and progesterone neither has an 11-oxygen they have side chains which are somewhat similar yet there are marked differences in metabolites

Dorfman The presence of oxygen at 11 changes the ratio in the C_{19} compounds but when there is a side chain and oxygen at carbon 11 the influence of the side chain seems to dominate the picture Certain C_{21} steroids may be metabolized to saturated C_{19} compounds These include substances like cortisone hydro cortisone 17α hydroxydesoxycorticosterone and 17α hydroxyproges terone All of these compounds have the characteristic feature of oxygen present both at carbon atoms 20 and 17 Such substances when administered to humans produce in part 17 ketosteroid by oxidative removal of the side chain These results are indicated in Table III Because the over all metabolism of C_{21} and C_{19} steroids is such that a ratio of 5β to 5α is approximately 9 and because it is known that substances like adrenosterone when metabolized to reduced ketosteroids yield ratios of the 5β to the 5α isomers of 0 2 it is unlikely that the only mechanism of conversion of C_{21} to C_{19} steroids is by removal of the side chain followed by reduction of Ring A If this were so the administration of cortisone and hydro-cortisone would yield ratios of the 5β to 5α isomers in the neighbor hood of 0 2 instead of the experimentally determined value in the order of 9 Further if substances like cortisone and hydrocortisone were fully reduced in Ring A before the side chain was removed one would expect a ratio of the C_{19} reduced forms derived from the C_{21} compounds to be present in a ratio of approximately 20 But that is not in agreement with the experimentally determined value of 9 Therefore it is suggested that actually two pathways for the conversion of C_{21} steroids to C_{19} steroids exist the minor pathway would involve the oxidative removal of the side chain with the formation from cortisone of adrenosterone and from

TABLE III

Metabolism of Δ^4-3 Keto C$_{21}$ Steroids to Saturated C$_{19}$ Steroids In Vivo* (Humans)

Steroid Administered	No of Experiments	Ratio 5β/5α† Mean (Range)
Cortisone	3	6 5† (4 3 9 1)
Hydrocortisone	3	11 0† (2 0-24 4)
21 Desoxycortisone	1	4 1†
17α Hydroxydesoxy corticosterone	2	11 8**(7 0 16 5)

Data of Burstein et al (9 10 11) Rubin and Dorfman (unpublished)

† 5β = 11 keto plus 11β hydroxyetiocholane 3α ol 17 one

5α = 11 keto- plus 11β hydroxyandrosterone

** 5β = etiocholane 3α ol 17 one

5α = androsterone

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hydrocortisone 11β hydroxy Δ^4 androstene-3 17 dione The major but not exclusive pathway would involve the preliminary reduction of the $\Delta^{4,5}$ double bond with the subsequent oxidative removal of the side chain The C_{19} metabolites derived from the two pathways one yielding a ratio of 0 2 and the other a ratio of approximately 20 would then make up the specific mixture resulting in a final ratio of 9 It has been estimated that approximately 88 per cent of the hydrocortisone and cortisone to be converted to C_{19} steroids probably go through the C_{21} 5β intermediate and that the remaining 12 per cent go through the Δ^4 3 keto C_{19} intermediate

From metabolic studies with various steroids in human subjects we can make an estimate of the percentage of administered steroid that is recovered or determinable in the urine The percentage of recoveries varies according to the type of compounds that we deal with For substances possessing the Δ^4 3 keto group in the $C_{19}O$ series a total recovery of ketosteroids of approximately 40 per cent may be expected For a substance such as adrenosterone a repre

sentative of the Δ^4-3 keto group of $C_{21}O_3$ compounds a recovery of 15 per cent is indicated In the C_{21} series compounds of the type of 17 hydroxy 11 desoxycorticosterone appear to be converted to C_{19} 17 ketosteroids to the extent of 5 per cent while hydrocortisone and cortisone are converted to ketosteroids to the extent of 3 per cent Actually we have used these figures in an attempt to calculate the specific production of individual ketosteroids in the body from data on quantitative excretion of related metabolites in the urine

Long That is total metabolite recovery?

Dorfman No these are ketosteroids In each instance there are other metabolites In the case of cortisone for example by far the largest amount by weight would be the tetrahydrocortisone of the 3α hydroxy 5β form

In the case of testosterone and Δ^4 androstene 3 17 dione there is a figure of 40 per cent other metabolites are present in minute concentrations which still have the steroid nucleus

Ralli Were these all administered to normal subjects?

Dorfman These figures are an average of our experience with various subjects including some rheumatoid arthritics

Bauer What percentage were rheumatoid arthritics?

Dorfman About 10 per cent for the whole experiment

I should like to go on now to indicate a method for the precise calculation of the origin of the 11-oxygenated C_{19} 17 ketosteroids in normal urine Of importance in this work is the utilization of a new method for the quantitative determination of six individual 17 ketosteroid components in urine by Rubin *et al* (17) Once these values are available together with certain preliminary figures on the generalizations which I have already presented it becomes possible to set up mathematical relationships so that the origin of the individual 11 oxygenated C_{19} ketosteroids may be calculated The data I shall use for these calculations are derived from analyses of the urine of normal men from 19 to 35 years of age The mean value for eight such subjects was found to be 1 08 mg per day for the 11 oxygenated compounds of the androstane (5α) series and 0 86 mg per day for the 11 oxygenated compounds of the etiocholane (5β) series Because we have already determined that cortisone and hydrocortisone can give rise to both 5β and 5α 11 oxygenated C_{19} steroids in a ratio of 9 1 and because adrenosterone and related compounds also are metabolized to both 5β and 5α forms in a ratio of essentially 1 1 it is possible to set up the following mathematical relationships

TABLE III

Metabolism of Δ^4 3 Keto C_{21} Steroids to Saturated C_{19} Steroids *In Vivo**
(*Humans*)

Steroid Administered	No of Experiments	Ratio 5β/5α† Mean (Range)
Cortisone	3	6 5† (4 3 9 1)
Hydrocortisone	3	11 0† (2 0 24 4)
21 Desoxycortisone	1	4 1†
17α Hydroxydesoxy corticosterone	2	11 8**(7 0 16 5)

Data of Burstein *et al* (9 10 11) Rubin and Dorfman (unpublished)
† 5β = 11 keto plus 11β hydroxyetiocholane 3α ol 17 one
5α = 11 keto- plus 11β hydroxyandrosterone
* 5β = etiocholane 3α ol 17 one
5α = androsterone

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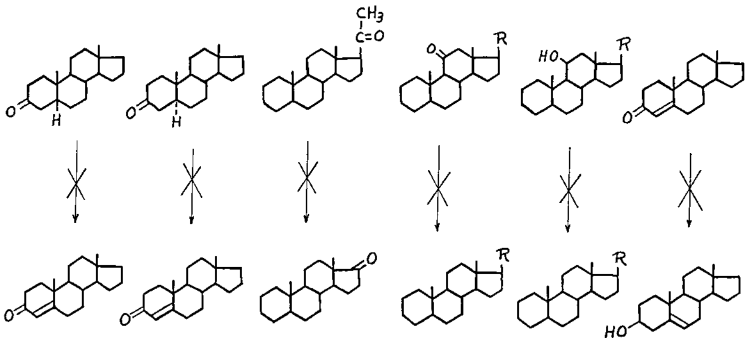


Figure 6 Reactions that do not appear to occur Reprinted by permission from Dorfman R I and Ungar F *Metabolism of the Steroid Hormones* Minneapolis Burgess Publishing Company 1953

Total 5α 11 oxygenated C_{19} steroids = 1 08 mg per day
Total 5β 11 oxygenated C_{19} steroids = 0 86 mg per day

Ratio $\frac{5\beta}{5\alpha}$ (for hydrocortisone and cortisone) $= \frac{9}{1}$

Ratio $\frac{5\beta}{5\alpha}$ (for adrenosterone and related steroids) $= \frac{1}{4}$

Let X = quantity of 5α from cortisone and hydrocortisone Y = quantity of 5β from cortisone and hydrocortisone

Then

1 08 − X = quantity of 5α from adrenosterone and related steroids

0 86 − Y = quantity of 5β from adrenosterone and related steroids

Ratio $\frac{5\beta}{5\alpha}$ (for hydrocortisone and cortisone) $= \frac{Y}{X} = \frac{9}{1}$

Ratio $\frac{5\beta}{5\alpha}$ (for adrenosterone and related steroids) =

$$\frac{0\,86 - Y}{1\,08 - X} = \frac{1}{4}$$

Solving for X and Y

X = 0 07 1 08 − X = 1 01

Y = 0 63 0 86 − Y = 0 23

In a similar manner it should be possible to calculate the origin of androsterone and etiocholane in normal men

Thorn Should we assume that there was no adrenosterone secreted directly as such?

Dorfman We cannot assume that As a matter of fact adrenosterone and/or 11β hydroxy Δ^4 androstene 3 17 dione is secreted

Thorn Am I correct in thinking that it is not necessary to know the total amount of cortisone in order to derive the significance which you attach to this ratio

Dorfman That is right Rather than show you all the reactions that occur in the organism I shall discuss the reactions that do not appear to take place These are illustrated in Figure 6 These facts actually simplify steroid metabolism (*in vivo*) and make it possible to relate urinary steroids to their tissue precursors Once the double bond at carbon 4 and 5 is saturated either as the 5β or the 5α in both the C_{19} series or in the C_{21} series the Δ^4 or Δ^5 group is no longer formed Specifically neither androsterone or etiocholane are transformed to Δ^4 androstene 3 17 dione

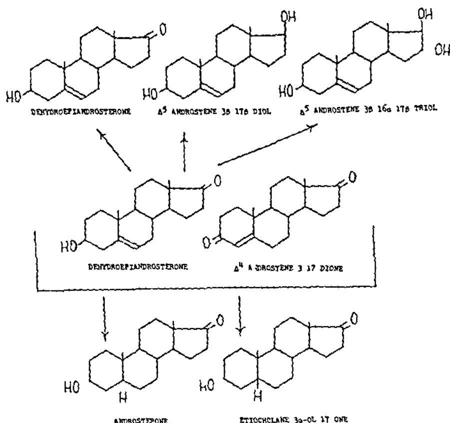


FIGURE 7 Differential *in vivo* metabolism of dehydroepiandrosterone and Δ^4 androstene 3 17 dione

the calculations previously suggested it should be possible to estimate the amounts of these steroids derivable from $C_{19}O$ compounds and those that come from the C_{21} steroids that we are at present considering Figure 8 outlines our particular problem It can be seen that the two steroids 17α hydroxyprogesterone and 17α hydroxy 11 desoxycorticosterone may be analyzed as a group by three independent measures first by using the two 17 keto steroids androsterone and etiocholane second by analysis of pregnane 3α 17α diol 20 one and third by analysis of pregnane 3α 17α 21 triol All three of these measures it should be pointed out indicate the combined production of the two C_{21} steroids One additional analysis would have to be done in order to measure each of these compounds The substance pregnane 3α 17α 21 triol 20 one would be a specific measure of 17α hydroxy 11 desoxycorticosterone

Further as far as we know and as far as others have observed one needs oxygen at carbon 17 as well as at carbon 20 in order to permit the conversion of a C_{21} compound to a 17 ketosteroid This fact also helps to permit the differentiation of 17 20 oxygenated steroids from 20 oxygenated steroids

Another point is that the presence of oxygen on the ring at carbon 11 does not yield the corresponding desoxysteroid in any appreciable quantity and finally that the Δ^4-3 ketone cannot be converted back to the Δ^5 3β hydroxy group This is particularly fortunate because it affords a method to differentiate and quantitate *dehydroepiandrosterone and Δ^4 androstene 3 17 dione*

I should like now to discuss with you various methods proved or indicated that would lead to a precise analysis of individual endogenous steroids produced by the adrenal cortex Our first problem consists in the differentiation of dehydroepiandrosterone and Δ^4 androstene 3 17 dione Both these substances on the basis of best available evidence are probably produced by the adrenal cortex Further it is known that part of the dehydroepiandrosterone produced in the organism can in fact be converted to Δ^4 androstene 3 17 dione Further both these 17 ketosteroids yield the common metabolites androsterone and etiocholane It is obvious therefore that some additional means would be needed to differentiate quantitatively between these two substances Figure 7 is presented to indicate such a possibility In other words both dehydroepiandrosterone and Δ^4 androstene-3 17 dione would be expected to yield the two common metabolites androsterone and etiocholane Because the Δ^4 3 ketone cannot be transformed in metabolism to the Δ 3β hydroxy group the C_{19} steroids still possessing this grouping should in fact be derived only from dehydroepiandrosterone Three such indicators in the urine are available *to us for consideration* These are *dehydroepiandrosterone* Δ^5 androstene 3β 17β diol and Δ androstene 3β 16α 17β triol The *quantitative measure of any or all of these* constituents would in fact be a measure of the endogenously produced dehydroepiandrosterone On the other hand the measure of androsterone and etiocholane would also be a measure of dehydroepiandrosterone and Δ^4 androstene 3 17 dione By noting the difference it should be possible *to evaluate quantitatively both of these* C_{19} *O androgens*

A similar problem is found with respect to the two C_{21} steroids 17α hydroxyprogesterone and 17α hydroxy 11 desoxycorticosterone We know that both these compounds can be converted to saturated C_{19} 17 ketosteroids androsterone and etiocholane By means of

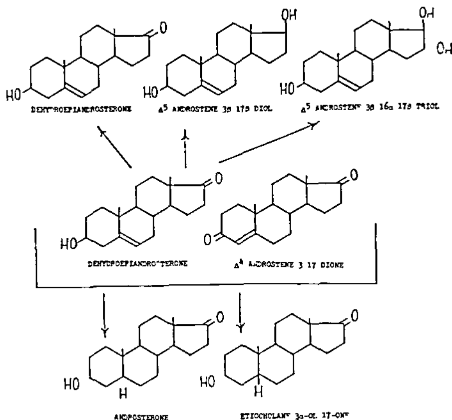


FIGURE 7 Differential *in vivo* metabolism of dehydroepiandrosterone and Δ androstene 3 17-dione

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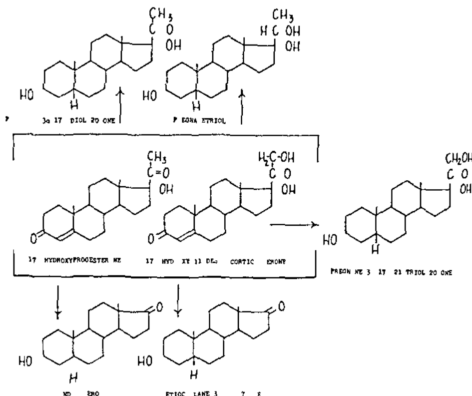


FIGURE 8 Differential *in vivo* metabolism of 17α hydroxyprogesterone and 17α hydroxy 11 desoxycorticosterone

This compound has already been shown to be a metabolite of the theoretical intermediate or dihydro form Once the concentration of 17α hydroxy 11 desoxycorticosterone can be calculated from an independent index the precise production of both constituents could be estimated

We go on now to consider the metabolism of the 11 oxygenated steroids *In Figure 9 we have represented the metabolism of both* adrenosterone and 11β hydroxy Δ^4 androstene 3 17 dione Both these substances are converted to four 11 oxygenated 17 ketosteroids as indicated It is not possible at the present time to differentiate between the 11β hydroxy and the 11 keto forms However as indicated earlier it is possible to calculate the endogenous production of Δ^4 3 keto 11 oxygenated $C_{19}O$ *compounds on the basis of* ratios formed in metabolism

Figure 10 illustrates the metabolism of cortisone and hydrocortisone and here we have multiple indicators First we may

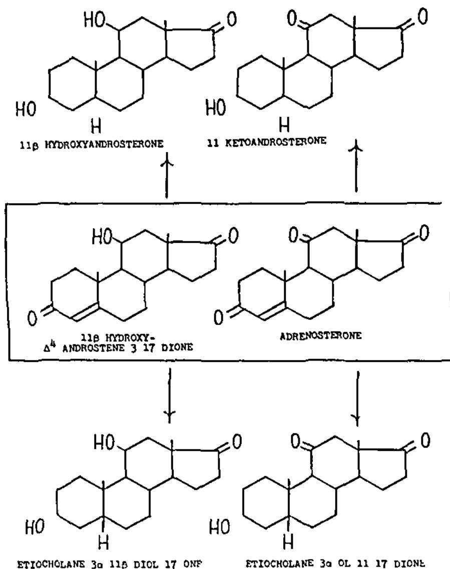


FIGURE 9 Metabolism of adrenosterone and 11β hydroxy Δ^4 androstene 3 17 dione

PREGNANE-3α 11β 17 21 TETROL 20-ONE

PREGNANE 3α 17 21 TRIOL 3 20 DIONE

PREGNANE 3 11β 17 TRIOL 20-ONE

PREG ANE 3α 17 DIOL

HYDROCORTISONE

CORTIS NE

11 HYD XY NO STER E

11 KETO NDROS ERONE

ETIO NOL NE 3 11β DI L 17 ONE

ETIOC OL E 3 L 11 17 TI E

Figure 10 Metabolism of cortisone and hydrocortisone

mention the four 11-oxygenated 17 ketosteroids which are similar to those derivable from the $C_{19}O_3$ compounds already discussed We have also considered the manner in which we can calculate the origin of the 11 oxygenated $C_{19}O_3$ compounds on the basis of ratio studies Specific indicators of cortisone and hydrocortisone are the unchanged substances but here again we cannot decide whether the endogenously produced material contains an 11 keto or an 11 hydroxy group because this group is interchangeable in metabolism and no generalization or hypothesis can be made as to the factors which influence the interconversions The tetrahydro derivatives of both hydrocortisone and cortisone are perhaps the most valuable indicators of endogenously produced cortisone and hydrocortisone These are excreted in the largest amounts perhaps as high as 6 mg per day in the urine of a normal adult An additional index of hydrocortisone and cortisone production are probably the 21 desoxytetrahydro derivatives but because their concentration is relatively low and methods for their detection are not established we shall not dwell on this point

Long Dr Dorfman has presented this difficult subject very clearly May I ask what is the spectrum of biological activity obtained with this variety of compound for instance following the administration of cortisone what type of biological activity would the urinary steroids exhibit?

Dorfman Cortisone (or hydrocortisone) is excreted as such and therefore we have adrenocortical activity the conversion to 11 oxygen androsterone derivatives means that androgens are excreted to a small extent There might be some inhibitors of adrenocortical hormones in the tetrahydro derivatives of cortisone but that has not been definitely established

Long What I have in mind is whether the administration of any of these steroids actually increased the amount of let us say androgenic material in the urine

Dorfman Cortisone yields some urinary androgens

Thorn We have done the androgen assays on cortisone but not the salt retaining assays

Young Is there any indication that steric hindrance can account for some of these differences particularly between 5-α and 5 β substances?

Dorfman I believe that the basic question in the 5β to 5α ratio is the enzyme responsible for each of the reductions

Young I think I am right in my belief that if cholesterol is reduced directly with the double bond in the 5-6 position we

obtain cholestanol which is 5α If we first oxidize the hydroxyl group to ketone so that the double bond is reduced in the 4 5 position we then obtain coprostanol which is 5β That also seems to occur in the intestine It appears to be related to the steric hindrance and perhaps also to the type of phenomenon that you are concerned with

Gallagher I think it is a question of the enzyme systems that are present I believe the reduction of these alpha beta unsaturated ketones *is biochemically a rapid and complete reaction The stereo*chemistry is a result of the enzyme systems that participate in them I do not believe the analogy can be extended from a platinum catalyst a hydride or other chemical reducing agent to the bio chemical system

Young However there is a difference with inorganic catalysts between the reduction of delta 4 5 and delta 5 6 substances

Bloch On the other hand biologically Δ^4 cholestenone is reduced to cholestanol to a significant extent

Young Coprostanol is the predominant steroid excreted in feces

Bloch Yes but if Δ^4 cholestenone is administered to rats it *can be shown that a high percentage of the reduction product is 5α*

Ralli Dr Dorfman is the starting point of the synthesis of all these compounds cholesterol?

Dorfman Cholesterol and acetate appear to be precursors of the neutral steroids

Ralli I ask this because Dr Gallagher mentioned the influence of enzyme systems on these reactions and I was wondering which one he had in mind

Gallagher I had in mind only those enzyme systems responsible for the conversions that Dr Dorfman has been talking about I was not concerned with the total synthesis

Ralli You mean the enzyme systems concerned particularly with acetate?

Gallagher Yes those concerned with the reduction of alpha beta unsaturated ketones and the oxidation reduction reactions of the 11 hydroxy group and the side chain

Ralli Do you have evidence that some enzymes will influence either the rate or the degree of this synthesis?

Gallagher No

Dorfman It seems quite likely that two enzymes are present one which catalyzes reduction to the 5α form and the other to the 5β form However at the moment we are doing studies on this

in vitro and I think I should point out that certain serious complications have been encountered such as a discrepancy between the *in vivo* and the *in vitro* metabolism

Long Where are these enzymes? Are they liver enzymes?

Dorfman Liver is a particularly effective source but the kidney also has some I suspect that we should also find them in other tissues if we were to look for them Certainly the enzyme systems concerned with the oxidation or reduction of the oxygen function at carbon 17 are widely distributed in the organism

Long Has it been established that the liver is the main organ in which these changes are taking place? What about the biliary excretion of the metabolites or the hepatic circulation of the steroids if the point of attack is largely in the liver? Do these products appear only in the urine after they have circulated through these organs?

Dorfman We really have no data on the fecal excretion of neutral steroids but I think Dr Gallagher does and will probably take up this question We have recently reported that when testosterone was administered to patients with biliary fistulas we were unable to detect either androgenic activity of true 17 ketosteroids in the bile These patients received as much as 900 mg per day

Gallagher I think there is a real species difference here Dr Long In the rodent with the radioactive steroids cortisone and testosterone there is a very considerable biliary excretion of either the hormone or the end products The principal route of elimination in the rodent is the gastrointestinal tract and as much as 70 per cent of the metabolites of these hormones are found there

In the human however this does not appear to be the case The principal route of excretion is the urine and in the case of these two substances fecal excretion is approximately 10 per cent of the dose administered over as long a period as seven days Usually it is nearer 5 per cent of the total amount of radioactivity administered

Long It is an interesting point that there should be this large difference between the species

Dorfman That is why we chose the human

Gallagher I do believe in contradiction to Dr Dorfman that some material does get into the bile in the human but it may be to a very considerable extent reabsorbed

Thorn Regarding the liver enzymes in some preliminary observations which we carried out two or three years ago large doses of steroid were given in the presence of diffuse acute liver disease such as hepatitis It was of interest to note that the patients

handled their cortisone or compound F about as well during the period of illness as they did later when they appeared to be completely recovered as measured by hepatic functional tests It thus appeared that either recirculation assisted in the metabolism of the steroids or some other mechanism was responsible for the change — a mechanism which does not seem to be interfered with by the liver despite widespread parenchymatous disturbance

Ralli Hepatitis presents a different problem from the point of view of the functional capacity of the liver, than does chronic liver disease

White Because Dr Gallagher has worked with labeled compounds in the human and because we have seen so little of the administered material accounted for in the nonlabeled experiments I wonder whether he could indicate where the rest of this material goes What is the fate of the labeled material if it does not go into the bile in man and if so little relatively, is found in the urine?

Gallagher I cannot provide any factual information One thinks immediately of course, of complete oxidation or total degradation of the steroid nucleus and the incorporation of the label into some other pool such as acetate or CO_2 The amount of material we used was so small that it was impossible to measure radioactivity in the expired air

Bloch Did you ever look into the acidic fractions?

Gallagher Yes of course There is some material there but the percentage is not large

White Has there been any opportunity to examine tissues?

Gallagher Certain of our studies bear on that point as well as the one that Dr Thorn mentioned a few minutes ago Cortisone t disappeared very rapidly from the synovial fluid indeed the disappearance of radioactivity was quite comparable to the disappearance of sodium ion in the same patient (Figure 11)

Bauer Was that a normal or an inflamed joint?

Gallagher A massive effusion so that samples of fluid could be withdrawn it was not an inflamed joint The sample of joint fluid withdrawn about 25 minutes after the introduction of the labeled cortisone had measurable radioactivity An isotopic dilution with cortisone was made and less than 5 per cent of the radioactivity was still present as cortisone Apparently either the enzymes in the synovial fluid or the membranes were able to metabolize that hormone and I suspect that this is also true of other tissues

Bauer There could be a very fast exit from such an articulation

Gallagher It was a rapid exit

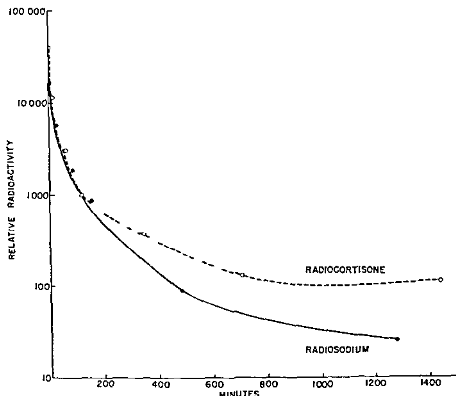


FIGURE 11 The disappearance of radiocortisone and radiosodium from the synovial fluid of a human subject Reprinted by permission, from Gallagher T F Biochemical problems of the steroid hormones *Chemical Specificity in Biological Interactions* No 3 Chapt V of Harvard Memoirs New York Academic Press 1954 (pp 50-64)

Bauer How much would be due to exit, and how much to oxidation?

Gallagher Of the material that remained in the joint fluid ap proximately 0 5 mg was still cortisone Perhaps that is not a tracer amount in the joint but it is still a small quantity There are limita tions in dilution even with radioactive tracers

Pincus Dr Vogt s (18) work on the fate of the corticosteroid activity coming from the adrenal has shown that there was scarcely a tissue which did not very rapidly "utilize the activity I suspect that the joint is merely a happy medium I think practically all attempts to trace corticosteroid in any tissues you may wish to mention will lead to a picture of very rapid disappearance

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Gallagher It was a rapid exit

Gallagher * The following figures will answer the question that was asked about the effect of ACTH on the excretion of these metabolites The subjects were two normal young university men The study was done by Dr Thorn and the urines were provided to Dr K. Dobriner I did not have much to do with the experiments except to bring them to completion

Figure 12 is concerned with Subject C-21 In the control period we may observe the 11 oxygenated steroids There is also another control period and three periods when 20 mg of ACTH were given intravenously each day There is a rise in 11 hydroxy androsterone of I think, very significant degree There is also a rise in 11 hydroxy etiocholane The interesting thing is that there is an increase and quite a marked one in the excretion of 11-desoxysteroids following this course of ACTH That is quite general and we have a number of instances of that response

We can contrast the situation after administration of compound E in a dose of 500 mg per day orally As Dr Dorfman has indicated the principal metabolite is a derivative of etiocholane 16 mg a day in the second period The formation of 11 hydroxy etiocholane indicates again as Dr Dorfman has pointed out that there is reduction of the 11 ketone group of cortisone There was a stepwise diminution of the excretion of 11 desoxysteroids

Figure 13 is the same type of study on another young man M 21 In this patient there is not quite so marked an increase in androsterone with ACTH a process which seems to vary in different subjects Invariably there is an increase in the etiocholane and in the 11-oxygenated compounds The cortisone results are much the same there is the same stepwise decrease of desoxy steroids and a marked increase in 11 ketoetiocholane

Bauer What about the 11 ketoandrosterone?

Gallagher We find the compound in small amounts and infrequently It is not an error in technique Were it present in any reasonable amount we would find it

I should like to ask Dr Dorfman what explanation he offers for the fact that the etiocholane metabolite of "F" or "E" is excreted largely as the ketone whereas the androstane metabolite is excreted largely as the 11 hydroxy compound

Dorfman Our results have also shown that in the 11-oxygenated etiocholane series the principal quantity of urinary material is in

*The following grants are gratefully acknowledged the American Cancer Society the Anna Fuller Fund the Lillia Babbitt Hyde Foundation the Teagle Foundation Grants (C-440) and (C-322) of the National Cancer Institute National Institutes of Health and the Damon Runyon Memorial Fund.

Selye Is there any difference in the disappearance of steroid between an inflamed and normal joint?

Gallagher We did not determine this We did not think we could withdraw enough fluid for satisfactory measurement from a normal joint that is why we took this very abnormal situation

Pincus We* have done the same experiment *in vitro* by taking joint fluid and incubating hydrocortisone What Dr Gallagher says was borne out very definitely Within half an hour the amount of hydrocortisone was reduced to a very small fraction of the amount originally incubated

Bauer Was the synovial fluid cell count high?

Pincus All specimens were cleared of cells by centrifuging

White Have you done any bioassays on such incubates after the hydrocortisone has disappeared

Pincus Only on glycogenetic activity which is very much reduced in this case

Ralli In the *in vitro* studies did you incubate with any other substances besides cortisone and the synovial fluid? Would it be possible to step up the oxidation? I am thinking of the influence of the enzyme systems that Dr Gallagher mentioned

Pincus It was not necessary Within half an hour less than 5 per cent of the hydrocortisone could be accounted for All the rest was something else

Ralli There is no way of inhibiting it either?

Pincus No We stopped those experiments because we felt we should go to the actual enzymes rather than concern ourselves with miscellaneous fluids We boil the fluid and then we obtain good recoveries

Thorn What happens in the presence of heat?

Kendall Nothing

Thorn Isn t there some degradation with heat? Does the compound still maintain its stability?

Kendall Yes

Mason One of the pharmaceutical companies prepares the solution by suspending the material in saline and autoclaving

Pincus We have assayed those solutions and the activity is there I should like to ask what happens when ACTH is administered have you done any calculations on that?

Dorfman We do not have suitable data as yet

Pincus If there is a predominance of one steroid type rather than the other it might give us a partial answer

*Romanoff E B and Pincus G Unpublished data 1953

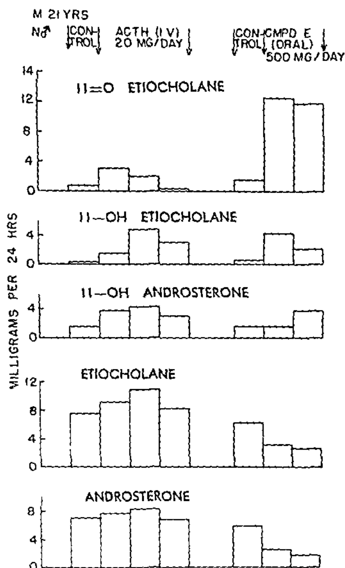


FIGURE 13 The effect of ACTH and cortisone upon the excretion of C 19 metabolites of steroid hormones in a normal man

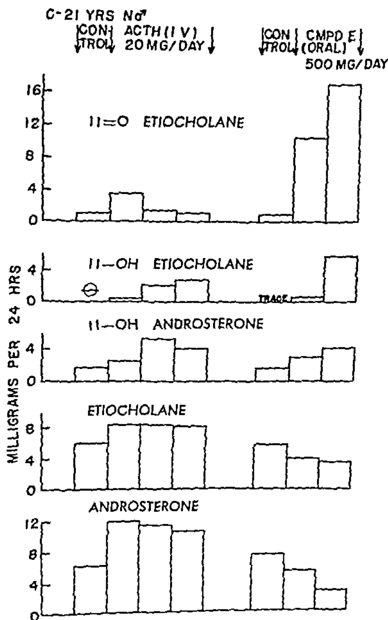


FIGURE 12 The effect of ACTH and cortisone upon the excretion of C 19 metabolites of steroid hormones in a normal man

Dorfman Administration of progesterone to the rabbit has been done with the isolation of the 5β stereoisomer pregnanediol

Gallagher That is still a small amount

Dorfman However, comparatively speaking it is not because in the normal human only from 10 to 15 per cent of the administered progesterone is obtained as urinary pregnanediol In the pregnant woman the conversion goes up as high as 30 per cent

White The rabbit is a very good case in point It is pretty well established from a variety of studies *in vivo* that the liver forms the 5 beta isomer from progesterone Have you done the counterpart *in vitro* with rabbit liver using progesterone?

Dorfman We have not done that yet

Long If the kidneys are removed in animals is there any possibility of measuring the rate of accumulation in the blood of these various products?

Dorfman I see no reason why it could not be done

Long I think you mentioned that the kidney may play an important part in changing these metabolites

Dorfman In experiments where both liver and kidney tissues were used certain enzymes have been found in both tissues Their concentrations appeared to be lower in the kidney than in the liver

Long Is it possible to make any estimate of what the half life of cortisone injected into the blood stream is?

Dorfman Dr Gallagher do you have some time studies on that?

Gallagher A short time after tracer amounts of less than a milligram of tritium labeled cortisone were injected intravenously we found a very small amount of radioactivity in the blood Further studies with hydrocortisone are in progress

Bulli What would you find in the kidney if you measured the radioactivity?

Gallagher The metabolites are rapidly excreted We have not looked in the kidney but have examined the urine The results I mention have been obtained with humans

Loeb Apropos of Dr Long's question what is known of the specificity of the renal clearance of these compounds? I think this would be of importance when dealing with such relatively small amounts as appear in urine I suppose it would be difficult to determine

Thorn We have had experience with the excretion of the tetra hydro compound where we obtained very high yields in the urine These ratios were compared with those obtained from hydrocorti

the 11 keto form while in the 11 oxygenated androstane series the 11 hydroxy form is major

Gallagher Are you speaking of normal people or after the administration of compounds?

Dorfman They are the values found in the urine from normal people I cannot explain these facts at present It may be the same sort of thing as the influence of the side chain, and the reduction of the double bond carbons 4 and 5

I should like to make some remarks with respect to the reduction of the C_{21} Δ^4-3 ketone by *in vivo* and *in vitro* methods I think it is well to point out at this time that there is a discrepancy which cannot be explained adequately If we administer any of the C_{21} Δ^4 3 keto compounds we obtain reduction products primarily of the 5β configuration However the reduced C_{21} compounds that have been isolated from tissues such as the adrenal are all of the 5α configuration If one incubates C_{21} Δ^4-3 ketosteroids with liver or adrenal tissues the primary and almost exclusive reduction products are in the 5α series Perfusion of both C_{19} and C_{21} steroids through rat and bovine tissues again yield 5α reduced steroids Thus at the moment we have *in vivo* studies on the human which yield for C_{21} Δ^4-3 ketones predominately 5β stereoisomers whereas *in vitro* and isolation studies indicate mostly 5α reduction productions

Thorn Do you have any experiments using the material recovered in the urine in experimental animals?

Dorfman The urinary excretion in animals at the moment is poorly characterized as compared to studies in humans We did some steroid metabolism studies years ago on the guinea pig We have isolated 5α stereoisomers in the C_{19} series and 5β stereoisomers in the C_{21} series These experiments were rather crude and the amounts isolated relatively low We are now approaching this problem in two ways (*a*) by observing *in vitro* steroid changes with tissue preparations and also *in vitro* steroid metabolism in the guinea pig and (*b*) by doing *in vitro* steroid studies on the human liver

Gallagher With respect to Dr Selye's question we made an attempt to study the nature of the metabolites after administration of testosterone to mice In both the urine and the feces we were unable to obtain the radioactive metabolites in either soluble form

Pincus There are a lot of data on the administration of progesterone in the rabbit

tion If they were both short we should anticipate that there would have to be a high rate of ACTH activity which continued for a long time On the other hand if the half life of cortisone were long we would only need to have a brief stimulus with ACTH and the effects from the adrenal hormones would be prolonged

Astwood Just because the substance is only there for a minute does not mean that the effect may not last for a long time

Long That is true but perhaps we should not speculate too far

Li Is it also possible that the hormone when injected into the body appears in a different form? Cortisone itself disappears in one minute yet it might be converted into some other substance and so escape detection

Long It should still have biological activity

Bauer And also pick up radioactivity

Long What I am trying to get at is this has anyone done an experiment of injecting cortisone into the blood and then sampling the blood at various intervals?

Pincus I believe the radioactivity experiment is the complete answer If there are any metabolites circulating in the blood radioactivity should be observed even if the metabolites are not identified

Thorn Since the conjugated material seems to disappear so rapidly from the blood stream one would anticipate that the biological activity would fall off in a matter of a few minutes

Ralli If it is true that the biological activity is decreasing so rapidly how do you account for its therapeutic effect?

Thorn Probably because the effect it produces metabolically persists after its initiation and requires time to return to the normal or low normal level We know for instance how rapidly a patient may escape from the salt retaining effect of a steroid when it is administered intravenously However if the period of administration has taken place over a period of time and if for instance the patient has accumulated an appreciable positive sodium chloride balance then it will require a number of hours before the kidney effectively excretes the accumulated sodium chloride Thus the patient would not show the signs of the disappearance of the sodium chloride retaining effect until the accumulated salt had been excreted and a negative balance had begun to develop

Ralli The trouble with that argument is that if what you are saying is true apparently the size of the dose would not affect the rapidity with which it is degraded It is being degraded at a very rapid rate

sone and desoxycorticosterone, endogenous creatinine excretion under fasting conditions forming the basis of reference

Bauer Leaf* has some data on humans but unfortunately it was obtained from individuals suffering from diabetic acidosis and extensive hemorrhage with reduction of renal function However apropos of Dr Long's question it would be of interest to do the same type of experiment on a heart lung kidney preparation it would not make it any easier to localize the site of action but it would provide a simpler system for study

Gallagher It seems to me with respect to the kidney threshold that for the hormones themselves this barrier must be appreciable There is little unaltered hormone in the urine On the contrary the kidney threshold for conjugated metabolites must be very nearly nonexistent because they are at such a low level in the blood stream and yet appear in the urine

Selye In connection with the question of the half life of corticoids and the much discussed problem of their possible "utilization" while they perform their antiphlogistic effects it is perhaps relevant to mention a few recent experiments performed with the "granuloma pouch technique which I outlined last year at our meeting

You will remember that in this procedure a definite cavity is formed under the skin by the injection of air and into this an irritant is introduced so as to produce inflammation We have now found that there is a definite critical period during which inflammation can best be prevented by a minimum amount of cortisone or hydrocortisone (19) Under our experimental conditions this critical period happened to occur 72 hours after introduction of the irritant It is well to emphasize that if the antiphlogistic hormone (e g cortisone) and the irritant are introduced simultaneously there is very little inhibition Similarly the injection of such hormones into the granuloma pouch after five or six days of exposure to croton oil is also quite ineffective in preventing inflammatory changes

I wondered whether the chemists of our group could not make use of this technique for the study of cortisone utilization It would be interesting to determine whether the disappearance and destruction of the hormone are actually greater during the critical period when it does some good than at other times when it fails to elicit any demonstrable actions

Long We know that ACTH has a half life in the circulation of only a minute or two Is the half life of cortisone equally short? If so it bears upon the entire problem of the rate of adrenal secre

*Leaf A Massachusetts General Hospital Boston Unpublished data

tion If they were both short we should anticipate that there would have to be a high rate of ACTH activity which continued for a long time On the other hand if the half life of cortisone were long we would only need to have a brief stimulus with ACTH and the effects from the adrenal hormones would be prolonged

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Ralli The trouble with that argument is that if what you are saying is true apparently the size of the dose would not affect the rapidity with which it is degraded It is being degraded at a very rapid rate

Thorn I believe that is true over quite a wide range of dose schedules If steroid is given as a single rapid intravenous administration in general a prolonged effect of the hormone will not be obtained unless of course a massive amount of material is infused whereas giving the same amount of material intramuscularly where it is absorbed more slowly, produces quite a marked prolongation of the physiological effect

Long In this connection there is one interesting experiment by Love (20) who injected epinephrine and immediately removed the adrenals just as fast as he could take them out and then followed the fall in the eosinophils He found he could not take the adrenals out fast enough to prevent the fall in the eosinophils once the gland had been stimulated This must have been due to the adrenocortical hormone put into the blood stream between the injection and adrenalectomy

Selye Do I understand correctly that Dr Gallagher feels the destruction of the corticoid is so rapid that such comparisons cannot be readily made?

Gallagher I did not use the term destruction I said disappearance It may be metabolized and excreted during that time

Selye What I mean is this in a granuloma pouch there is a closed cavity in which the lining is inflamed If hormone is injected into it during the critical period the inflammation will be inhibited but if it is injected at any other period the inflammation will not be inhibited I wonder whether the long debated question concerning the utilization of hormones for the performance of their activity could be successfully analyzed with this technique by comparing the rate of destruction during a given time interval for example at the critical period when the hormone is effective and at another time when the hormone is not effective

Bauer The physiological state is quite different in the two types of experiments that you relate In your initial experiment in which the two agents are injected simultaneously there is good lymphatic drainage whereas once inflammation is induced there is some obstruction of lymphatics If they leave by way of the lymphatic rather than the vascular system it would not tell you very much It might be another way of measuring lymphatic drainage

Selye I do not think this is very likely because we are giving hydrocortisone acetate in the form of crystals and the crystals remain there for days they may be seen by simple inspection

Bauer However your fluid exchange in these experiments is dependent in part upon the potency of the lymphatics in the one

instance they would be relatively normal in the other there might be considerable blockage of the lymphatics

Pincus One of the interpretations is that a prophlogistic substance is brought in early by the blood stream With the blockade of that area or of the lymphatics the hydrocortisone is present in relatively excessive amounts during the critical seventy two hour period I do not know what might happen after that period

Thorn May I go back to Dr Ralli s original problem If a free form of the hormone is injected intravenously there are not many physiological mechanisms which are sensitive enough to be measured significantly by hourly titration One of these however would be the renal excretion of electrolytes We know that within two hours one notes a reduction in the level of sodium excretion with a constant infusion of sodium and of hormone One must use a constant infusion of sodium or else it is difficult to interpret the falling off of sodium excretion if hormone is given intravenously without additional sodium chloride However with the constant infusion of sodium and hormone and the considerable retention of sodium one ultimately builds up a pressure head in which one observes an increasing escape from the effect of a constant level of hormone as measured by a gradual rise in sodium excretion On the other hand the moment one discontinues the hormone infusion there is a much more rapid rate of escape and an increased excretion of accumulated sodium

Ralli If you were going to study renal clearances that would be the ideal way to do it At the time you were infusing a constant amount of the hormone simultaneous clearances could be done for the hormone as well as for sodium chloride

Selye In connection with the remarks of Drs Bauer and Pincus I should like to repeat that in my opinion it is very unlikely that lymphatic blockade would be responsible for the observed effect Hydrocortisone proves to be most effective during the critical period even when it is administered systemically at a distance from the pouch so that there can be no question of it being "trapped at the site of inoculation in the pouch

Bauer When it is given parenterally during the first 72 hours the entrance into the pouch will be more rapid than it will be later because any acute inflammation permits a more rapid rate of entrance In the early stage of inflammation there may be no lymphatic blockage

I am speaking of the articular cavity we have had a limited experience with artificial cavities of the type you mention

Long What is known of the fixation of the corticosteroids by the tissues?

Gallagher We have attempted to study the question in mice (21) We came to the conclusion that the time cortisone resides in tissues in measurable amount must be a matter of minutes We did indeed find a slightly different tissue distribution within the first three minutes or as soon as we could kill the animals after intravenous infusion There appeared to be more radioactivity in the skin and the carcass than ten minutes after the administration of the hormone Certainly after an hour we do not find any localization in any organs except the liver and the gastrointestinal tract

Thorn Probably the most sensitive indicator in man is the Addisonian patient who has had no cortisone for several days prior to the study If under these circumstances a small constant infusion of cortisone is given over a 48 or 72 hour period one observes that equilibrium or at least a constant rate of excretion of hormone is not attained in the first 24 hour period This suggests that in the Addisonian receiving minimal doses of cortisone there is a pool which needs to be filled in the tissues before equilibrium of excretion is attained In normal subjects with intact adrenals given 100 mg of hormone intravenously one does not attain equilibrium by the end of an 8 hour period of infusion of hormone at the rate of 12 5 mg per hour

Gallagher The metabolites of hormones are rapidly eliminated as evidenced in Figure 14 Perhaps you will question the amounts we have given and I should not disagree with you During zero to five hours 16 mg of labeled testosterone are infused (22) That may be too large an amount possibly it is However while the infusion is in process more than a third of the material infused is in the urine in the form of definite and recognized metabolites

Thorn Is this instantaneous administration?

Gallagher No the hormone is given as evenly as possible over a period of four hours

Pincus Dr Gallagher I think the problem may be something like this there is a certain retention of radioactivity so over a period of days a tiny amount of material begins to come out What is it and where does it go? Is that the only really active material and is the remainder of little significance?

Gallagher I shall debate it but I do not know the answer 16 mg may be too much to give in that period of time The reason we chose that dose was to compare it with other experiments and permit the use of both C^{14} and deuterium as tracers We have since

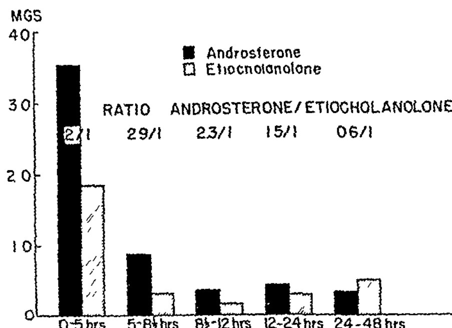


FIGURE 14 Excretion of androsterone and etiocholane in the urine of a man (Subject F) at intervals after a single injection of 16 mg of testosterone-4-C^{14} Reprinted by permission from Gallagher T F Bradlow H L Fukushima D K Beer C Kritchevsky T H Stokem M Eidinoff M L Hellman L and Dobriner K Studies of the metabolites of isotopic steroid hormones in man *Recent Progr Hormone Research* 9 411 (1954) (Academic Press)

repeated the studies with tracer doses but have not finished the work as yet

Pincus The only clue we have for this sort of thing is in inunction or local application experiments such as an anti inflammatory test in the mouse and according to Dougherty (23) hydrocortisone would be active locally in a very small fraction of the microgram Is that correct?

White Yes

Bauer That is not surprising if one consider it in terms of molecules instead of milligrams

Pincus I do not think it is surprising The point I am trying to make is that perhaps the active material which is localized is so small that ordinarily we cannot measure it I think that may be the answer to Dr Gallagher's experiment After all there is a limit to the amount of radioactivity that can be measured in tissues

Long Dr Gallagher if you could do the experiment again would it be worth trying something similar to what Stadie (24) has

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Gallagher I shall debate it but I do not know the answer 16 mg may be too much to give in that period of time The reason we chose that dose was to compare it with other experiments and permit the use of both C^{14} and deuterium as tracers We have since

Ralli Very large doses were given so that there would be enough material to analyze You also speak about the infinitely small amount that is needed to start these reactions When you give large doses are you measuring what goes on under normal circumstances?

Dorfman To answer that partially I should say that although we have worked with doses of hormones from 50 to 500 mg we have not as yet seen any qualitative differences in urinary products Actually in many instances we have even observed comparable quantitative figures for individual constituents

Long Can you make a kind of rough calculation on what these small amounts are? I think you gave us a figure this morning for cortisone or hydrocortisone production of around 20 mg a day as being necessary to maintain an adrenalectomized individual That is a rate of secretion of about a milligram an hour and if that is put into 50 kilos of body water obviously the effect of concentration any time during the hour is very low Assuming equal distribution when 500 mg is given it is at least 25 times the effective blood concentration since the quantity in the blood at any one time of a normal individual under ordinary circumstances is very low indeed A rate of production of about a milligram an hour is sufficient t maintain that quantity

Thorn There are two comments I might make First there considerable evidence that the normal individual does not sec hormone at the rate of a milligram per hour throughout th hours The active period of secretion appears to be concentr quite heavily in the 12 hours of the day Secondly if one give Addisonian patient a milligram per hour of hormone around t clock the physiological reaction is very intense and quite diff from that following the single administration of 25 mg once

Gallagher If I understand what you say you believe that t 20 mg per day estimate of production is a high value Do believe that it is smaller than that amount?

Thorn I would assume that the gland puts out at least 20 or more of hormone per day but that the secretion of this mate is concentrated in a very short period of the day and therefor physiological effect is appreciably less than if the material w secreted evenly throughout the 24 hour period This emphasize the point that it is not enough to know the total secretion of the gland in order to estimate its physiological effect one must als know the time during which that secretion has occurred bec that also has a very important effect on the total physiological action The ratios which Drs Jenkins and Laidlaw in our laborator

recently done with the isolated diaphragm where it is immersed in the solution for a short period of time and then washed free of all materials attached to it so that the amount of these materials can be determined? Stadie used this very successfully in the demonstration of the fixation of insulin

Thorn We thought that with the radioactive compound F one might study an adrenalectomized patient or animal given a very minute quantity of the substance so that one might analyze return blood flow from various organs and areas of the body in an attempt to trace the fixation or localization of the initial dose of hormone

Bloch From the fact that the 9 chloro compounds are 3-4 times more active than E or F could one not argue that conversion to more active forms occur?

Gallagher That can be interpreted otherwise of course I should suspect that the chloro compounds are more active because they are less readily metabolized

White In a local area where a good measurement of F or E like activity can be obtained it ought to be possible with micro-techniques of identification of E or F to ascertain whether there is histological evidence of persistence of activity at a time when there is no microchemical evidence of the existence of either E or F

Pincus We touched upon that earlier in discussing the disappearance of F *in vitro* from joint fluid It would not answer the question we are discussing because what is active is a very small amount of material and what one measures may be the excess It is a very difficult problem I think the Stadie technique is very much more definite

Ralli In Figures 12 and 13 I think Dr Gallagher presented the injection of substances in normal human subjects I notice cortisone was given and the effect of ACTH on the secretion of certain substances was compared to that of cortisone You gave 500 mg of cortisone a day to these normal subjects?

Gallagher Dr Thorn did

Ralli That is a rather large dose is it not?

Thorn Yes I should say so

Dorfman It was because of Dr Thorn s experiments that we were able to do most of our early studies on cortisone

Thorn The dose was calculated from the chemists expectations of the amount of material which might be recovered based on the known small proportion of material which would be likely to be excreted

be adequate for this purpose Of course one does not like to give a larger dose because then the continued action would carry over to the next day s study From our study on the Addisonian patient under constant hydrocortisone infusion we conclude that the diurnal variation which we observe in normal individuals is probably the result of alteration in secretory activity of the gland rather than a constant 24 hour secretory activity with alteration in metabolic function let us say in the liver and kidney

Bauer You therefore conclude that diurnal variations require adrenal glands?

Thorn Yes we believe that the diurnal variation requires a marked change in secretory activity of the gland We are particularly interested in the fact that although this variation is very sensitive and we are able to pick it up easily with our present methods of study this contrasts with the remarks I made a year or two ago when we pointed out that rather severe stresses do not produce a marked change in the secretory activity of the gland as measured by our methods Thus it is interesting to speculate on the magnitude of the diurnal variation and its sensitivity throughout the 24 hour period and yet its resistance is to a large extent to externally imposed stress during the 24 hour period

Bauer How much variation do you find in the secretion of the Addisonian patient or an adrenalectomized individual?

Thorn We give our hormone cyclically and of course the individual eats cyclically Both of these factors would tend to establish some rhythm in the Addisonian patient under ordinary treatment We are at present trying to establish the level of hydroxysteroid excretion in an Addisonian patient maintained on a more constant regimen throughout the entire 24 hour period

Astwood I think Dr J D Rosenbaum (25) demonstrated that there was a diurnal variation in water and salt excretion?

Bauer Would the diurnal variation in the Addisonian patient be the same as in the normal person?

Thorn One might anticipate a diurnal variation in water and salt excretion in adrenalectomized patients or patients with Addison s disease who are untreated since there are other known factors which modify the excretion of salt and water and which presumably would be functioning under these circumstances

Ralli I wondered whether Dr Pincus had any data on the diurnal variation in the mentally disturbed or agitated patient

Pincus We published something on that several years ago It is quite different from the normal but there are individual variations

have obtained would indicate that in general three times as much hormone may be secreted during the period of 7 00 a m to 1 00 p m and from 1 00 p m to 5 00 7 00 p m as occurs overnight

Ingle Dr Thorn, may I ask a question that is a bit off the subject? There are cyclic changes in the level of eosinophils throughout the day that have been attributed to cyclic changes in the release of hormones by the adrenal cortices Have you done the experiment of giving adrenalectomized or Addisonian patients a constant intake of steroid by continuous injection and then following the eosinophils to determine whether that cycle is abolished?

Thorn I am not as certain about the eosinophil cycle in the Addisonian patient as I am about the excretion of steroid in the urine With respect to the latter we know that the diurnal variation of hormone excretion is minimal when a constant infusion of cortisone or hydrocortisone is given intravenously over a 48 or 72 hour period in an Addisonian patient We also know that the eosinophil fluctuations are much less in the Addisonian than in the normal person We do not as yet have adequate data on whether or not a constant infusion of cortisone completely stabilizes the eosinophil level throughout the 24 hour period in an Addisonian patient

Loeb When you say diurnal variation in excretion of material which material do you have in mind?

Thorn Total 17 hydroxycorticoid and 17 ketosteroid as well

Bauer Do you observe a diurnal variation when you have given a constant infusion for 24 hours?

Thorn The normal pattern is one of high secretion from 7 00 a m to 7 00 p m and of low secretion from 7 00 p m to 7 00 a m The cut off points do not correspond to these hours but these 12 hour periods show the major day to night variation We are interested in investigating people who are working at night to see whether the diurnal variation is altered by this type of activity ACTH administered in the evening will increase the night secretion and excretion of hormone and can reverse the day to night ratio The constant infusions to which I referred were constant infusions of hydrocortisone in an Addisonian patient where one can eliminate spontaneous variations in endogenous secretion from the adrenal

Bauer The patient is on constant infusion?

Thorn No the 3 1 ratio day to night preponderance is a phenomenon which we observed in normal individuals It can be altered under severe psychological stress It is not affected appreciably by a 24 hour starvation period but can as I said be modified by the administration of ACTH Four or five units of the ACTH gel may

be adequate for this purpose Of course one does not like to give a larger dose because then the continued action would carry over to the next day s study From our study on the Addisonian patient under constant hydrocortisone infusion we conclude that the diurnal variation which we observe in normal individuals is probably the result of alteration in secretory activity of the gland rather than a constant 24 hour secretory activity with alteration in metabolic function let us say in the liver and kidney

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Ralli I wondered whether Dr Pincus had any data on the diurnal variation in the mentally disturbed or agitated patient

Pincus We published something on that several years ago It is quite different from the normal but there are individual variations

There are some individuals who show good diurnal rhythms and others who show very poor ones Among psychoneurotics particularly, there are individuals who show no diurnal changes They go right along as if they were secreting at the same rate morning and night

Bauer Even though they sleep well?

Pincus Yes The psychiatrists suggest that these are people who have rather interesting dreams

Thorn Dr Dorfman in view of what you said earlier one might anticipate that in emaciated patients a low 17 hydroxysteroid excretion and a low 17 ketosteroid excretion could be easily explained Recently however we encountered a patient who had a high 17 ketosteroid excretion and a low total 17 hydroxysteroid excretion by the method which we employ However when given ACTH this patient showed a completely normal response in terms of a marked increase in 17 hydroxysteroid and 17 ketosteroid excretions and furthermore, when given a known quantity of hydrocortisone the anticipated excretion of 17 hydroxysteroids in the urine occurred I wonder what your explanation might be for this unusual picture of high 17 ketosteroids and low 17 hydroxysteroids in a female patient

Dorfman If you had some of those extracts it would be very interesting to study the difference in this particular patient between 11 oxygenated and the 11 desoxyketosteroid That would be an important fact to establish Did she have any signs of hirsutism or masculinization?

Thorn No

Dorfman The fact that 17 hydroxysteroids are decreased would indicate that the hydrocortisone production should be decreased which would mean in turn that the 11 oxygenated compounds of etiocholane type coming from the C_{21} compounds should also be reduced Thus this would suggest that the precursors which would be increased in this particular individual would be the $C_{19}O$ (Δ^4 androstene 3 17 dione) or $C_{19}O_3$ (adrenosterone) type An analysis of the individual constituents would give us a more precise understanding of the problem

Thorn That is interesting because given a test dose of F this patient excreted the predicted ratio of 17 keto to 17 hydroxy so it did not seem to be primarily a shift in the metabolism I was wondering what your thought was on the secretion of the adrenal at that time

Gallagher May I ask why you measured ketosteroids in this woman?

Thorn Yes This was a long term study of a patient whose physical appearance was normal but who was psychologically disturbed and we were interested in observing the reaction of her adrenals at that time Later on it was of interest to note that she spontaneously reverted to the more normally expected 17 hydroxy 17 keto ratio in the urine

Pincus What were the ovaries like?

Thorn We do not know because no operation was performed We saw the reverse of this situation in other severely stressed individuals in conjunction with the studies of Dr Francis D Moore * of the Peter Bent Brigham Hospital Boston on patients after serious burns or major surgery In these cases we observed a maintenance at a high level of 17 hydroxysteroids with an initial rise in 17 ketosteroids and a subsequent reduction in 17 ketosteroid excretion to normal or low normal levels with a persistent high level of 17 hydroxy secretion This is the first time that we have observed a reversal in the opposite direction and a spontaneous recovery

Ralli Dr Pincus have you determined these substances in the disturbed individual before and after electroshock therapy which can arrest the agitation and return the patient within 48 hours to a completely relaxed state?

Pincus We have not studied that in detail

Dorfman Within 24 hours after electroshock we have seen increases in both 17 ketosteroids and corticoids determined by the formaldehydogenic method

Ralli When the patient comes into the quiescent phase what happens?

Dorfman We have never studied them at that time All we know is that therapeutic shock certainly calls forth definitive increases within a 24 hour period

Ralli As compared to excretion during the control period before therapy?

Dorfman That is right

Thorn Since this patient showed the normal anticipated response

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* Moore F D Steenburg R W Ball M R Wilson C M and Myrden J A Studies in surgical endocrinology I The urinary excretion of 17 hydroxycorticoids and associated metabolic changes in cases of soft tissue trauma of varying severity and in bone trauma (In preparation)

to the administration of cortisone and hydrocortisone, it is difficult to assume that there was a metabolic aberration at this time accounting for an altered metabolism of the steroids once secreted Since she showed the normal response in 17 hydroxy and 17 ketosteroid values following ACTH the gland was able to respond normally to exogenous ACTH That was why I was interested in Dr Dorfman s explanation of the physiological significance of the altered ratio

Dorfman I cannot explain the ACTH stimulation data in this patient unless the amount of ACTH she secreted was at a minimum level — enough for certain reactions to proceed but not others When ACTH is administered essentially all the necessary enzymes are brought into play

Thorn You suggest that a qualitative difference in the ratio of 17 hydroxy and 17 ketosteroid in the urine might be due to a quantitative difference in the level of ACTH secretion

Dorfman That is right

Long When large quantities of these steroids are administered do any appear in the spinal fluid?

Dorfman I do not know of any evidence for or against that

Thorn I believe studies have been made on this point although we have not done any of them We were interested in the problem however during our experiments on the effect of ACTH and cortisone in patients with long standing multiple sclerosis

Pincus In regard to our problem Dr Oscar Hechter has some data which may be pertinent * He has been giving rabbits ACTH and then sampling the adrenal vein blood A rabbit is an animal which if you just take it as it comes secretes mostly compound B corticosterone and after the initial ACTH administration there is an increase in B output However after a matter of weeks of administration compound B practically disappears and only compound F is found Thus I think there is a possibility that a qualitative change may occur under such circumstances However this is an extreme instance

Thorn If you believe that the low dosage of ACTH gives rise to this abnormal ratio are you able to account for the fact that patients with inanition who have low 17 hydroxysteroids also have low 17 ketosteroids?

Dorfman In inanition as I recall the evidence tends to indicate a normal corticoid excretion by the older methods as compared to the 17 ketosteroid which do significantly decrease

*Kass E Hechter O Macchi I A and Aycock H Unpublished data 1953

Actually it would be a reverse picture of this After a relatively intense stress such as a severe burn the corticoids stay up for about five days while the 17 ketosteroids go up immediately and are below normal within one to two days The latter do not return to normal for about 10 to 12 days We observed that in our early studies with burned and infected patients in collaboration with Dr Shipley (26) This was a period when many investigators showed this type of diagram and we obtained it too However after that we ran a number of these experiments and did not always see it and I think I should agree with Dr Gallagher that it is not an invariable pattern Perhaps it depends on the type of stress duration and other circumstances so it warrants further study I think

Conn We have been interested too in the stress pattern and generally have obtained the elevation of both types of steroids initially This is followed by a decrease in the 17 ketosteroids with the 17 hydroxycorticoids remaining elevated for the longer period of time An interesting thing happens in patients with chronic liver disease Administration of steroidal compounds that increase 17 ketosteroid excretion in normal people fails to increase significantly the 17 ketosteroid excretion in patients with this disease We had an opportunity to study urinary steroidal excretions in a patient with severe liver disease on whom major surgery was performed The operation resulted in no increase at all of urinary 17 keto steroids but we observed the highest values for formaldehydogenic steroids which we have ever obtained in our laboratory This suggests that much of the increase of urinary 17 ketosteroids observed after severe stress is derived from 17 hydroxycorticoids and that this conversion is a function of the liver

This again brings up the point regarding some of the enzymatic activities of the liver versus the kidney in this respect In the case cited the liver was abnormal the kidneys were presumably capable of making the transformations but did not Also we may have a partial answer to the question Dr White brought up with respect to diurnal variation in the eosinophils in Addisonian patients receiving a fairly constant amount of cortisone We did not administer the cortisone intravenously as Dr Thorn did but we gave 5 mg orally every four hours throughout the 24 hour period and found no diurnal variation in eosinophils under those circumstances In addition we applied the stress of exercise in an effort to see whether exercise superimposed on those circumstances might induce eosinopenia However it failed to occur under those circumstances

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of this disease by saying that these individuals do not have a demonstrable adrenal cancer nor do they show significant masculinization. We shall consider the overt signs of overproduction of androgenic substances and the absence of any significant amount of metabolic disease due to overdosage of hydrocortisone. Finally our classification of adrenal hyperplasia (adrenogenital syndrome) will refer to those patients who show typical masculinization but are free of cancer and who do not reveal the metabolic effects of excessive amounts of hydrocortisone and cortisone.

Thorn: For purposes of clarification would you be willing to change your classification and make Cushing's syndrome represent bilateral hyperplasia so that we do not have both clinical syndromes and anatomical characterizations as the basis for your classification? This might be quite confusing in our subsequent discussion.

Dorfman: Let me present them as I have it and then you will tell me where corrections are necessary.

Loeb: I think most of us would call the "adrenogenital syndrome" your final item?

Dorfman: Yes, "adrenogenital syndrome" would be a better term than "adrenal hyperplasia."

As a background for our consideration of the biochemical differentiation between the different types of adrenal hyperactivity it may be profitable first to consider the normal adrenocortical hormone production. The events on the basis of the best available information plus of course some speculation are listed in Figure 15. The thicknesses of arrows indicated in this figure should serve as guides for the following three figures numbered from 16 to 18 in which changes or modifications in amounts of hormones at various points are indicated. Although the relative thickness of these lines are not considered to be absolute they are indicative of the relative amounts of steroids produced at any given level.

Starting with the pituitary control of the adrenocortical hormone production we consider ACTH as a crucial stimulus acting upon the adrenal cortex in two distinct manners. ACTH (either one or more principles) is responsible for the growth and maintenance of the adrenal cortex and also is concerned with the transformation of cholesterol and smaller particles to the steroids which are eventually converted to adrenocortical hormones (C_{21}) and androgenic hormones (C_{19}). Δ Pregnene 3β ol 20 one (pregnenolone) and dehydroepiandrosterone are considered to be these "first" sub

Thorn However I believe that you might have obtained it if the stress had been severe enough We have seen eosinopenia develop *in patients with Addison's disease when there had been acute* serious illness prior to the administration of cortisone

Conn Yes, but under those conditions the eosinopenia may have *nothing to do with the presence or absence of cortical hormone*

Thorn That is right We did some experiments on a telephone operator who did not show the expected reversal and rhythm He was on the schedule six nights per week, with one night off However the night telephone operator's activities decrease after 1 00 or 2 00 a m so perhaps this particular individual would not give us the greatest difference in day to night activity This subject must be investigated with further studies

Pincus We have published some reports on the 17 ketosteroid rhythms of people working in a factory at night Under these circumstances the excretions were reversed Their 17 ketosteroids were maximum whenever they awoke Whether it was half an hour before they went to the job or several hours before I personally went through a period of insomnia During the first night I did not show much change but during the second I did

Thorn The point I wish to make is that the rhythm is not easily interfered with and it certainly is not altered by the first night one remains up all night Thus the diurnal variation does not appear to be directly related to general activity or change in food habit but undoubtedly it can be reversed over a more prolonged period of alteration in the character of day to night activities This point however certainly needs clarification

Long We shall continue the discussion by asking Dr Dorfman to give us his ideas on the metabolism of adrenal steroids in certain pathological conditions

Dorfman I should like to present at this time working hypotheses for the study of the biochemical defects that are associated with various types of adrenocortical hyperactivity We shall consider the clinical entities of Cushing's syndrome adrenal cancer and the adrenal hyperplasia (adrenogenital syndrome) Since many mixed types of these diseases are known it is obvious that our speculations will not pertain to all the possible variations However it may be profitable to consider these three classes of adrenal hyper activity individually to indicate biochemical defects By Cushing's syndrome we mean that type of adrenal hyperactivity which is characterized by the metabolic changes arising from overdosage of hydrocortisone and/or cortisone We shall further limit our discussion

stances which result from the precursors cholesterol and/or smaller particles as a result of ACTH action

It is suggested that there are two principal pathways of steriodo genesis in the adrenal gland one concerned with the C_{21} compounds and the other with the C_{19} steroids The possibility of movement directly from the C_{19} to the C_{21} and back is indicated by arrows in Figure 15 with question marks This interrelationship although possible does not appear to be important Once a substance such as pregnenolone in the C_{21} series is produced it is subjected to the enzyme (3β dehydrogenase) which has the ability to oxidize the Δ^5 3β hydroxy group to the Δ^4-3 ketone thus forming progesterone A similar reaction occurs in the C_{19} series when dehydroepiandrosterone is oxidized to Δ^4 androstene 3 17 dione The next reactions in the biosynthetic sequence are those of hydroxylation In the C_{19} series the only hydroxylation reaction that we shall consider is 11β hydroxylation and the product formed from Δ^4 androstene 3 17 dione is 11β hydroxy Δ^4 androstene 3 17 dione which in peripheral metabolism is converted to a variety of 11 oxygenated 17 ketosteroids and has already been discussed In the C_{21} series hydroxylation of progesterone can yield six different types of hydroxylated products Not all of these possibilities are listed in Figure 15 Actually hydrocortisone in the human accounts for the principal biological activity (carbohydrate protein stress etc) and it is probably this substance which exerts the principal inhibitory influence on the anterior pituitary The production of hydrocortisone from progesterone involves three types of hydroxylation namely 11β 17α and 21 The combination of 11β and 21 hydroxylations on progesterone result in the formation of corticosterone The 21 hydroxylation of progesterone results in the production of desoxycorticosterone which is not listed in Figure 16 Other compounds are possible such as the Δ^4 pregnene 11β 17α diol-3 20 dione appearing in Figure 16 which actually represents a 21 desoxyhydrocortisone but of great importance is the eventual formation of hydrocortisone resulting from the hydroxylation at all three positions in progesterone

The control or level of activity of the pituitary adrenal system is in effect controlled by the amounts of ACTH produced by the anterior pituitary and the amount of hydrocortisone acting back on the anterior pituitary to control the ACTH production This suggested scheme has a very important implication a direct mutual control between the anterior pituitary and the formation of hydrocortisone and substances possessing adrenocortical hormone activity

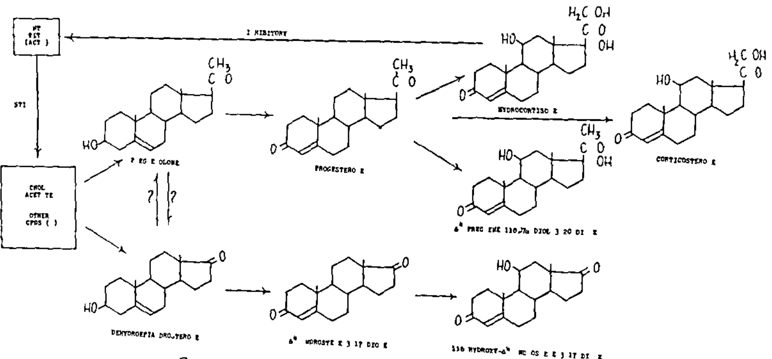


Figure 15 Adrenocortical steroid production by the normal gland

but an indirect control of the androgen production In other words androgen production is dependent upon the quantity of ACTH produced by the anterior pituitary and the amount of hydrocortisone that eventually is produced as a result of the ACTH stimulation This point is of great importance as will be noted later in our discussions on adrenal hyperplasia (adrenogenital syndrome)

White You are disinclined at this stage to draw a line between the two extremes from pregnenolone on down?

Dorfman Yes between the C_{19} steroids and C_{21} steroids I am suggesting two pathways

White You are still supporting the idea you had in the past that these are two separate pathways rather than a single common route of metabolism?

Dorfman Yes I believe in the relative independence of the two pathways If this scheme presents the true picture a unique situation arises The androgenic production by the adrenal is controlled only indirectly and is dependent upon the relationship between hydrocortisone and ACTH production by the pituitary gland I think that leads up to our basic story

Gallagher Do you believe that corticosterone does not influence the pituitary?

Dorfman Both hydrocortisone and corticosterone (and other steroids) probably influence the anterior pituitary but the relative pituitary inhibitory activity is such that normally the concentration of hydrocortisone determines the issue

I should like to continue now with a consideration of the steroidogenesis in the condition of pure Cushing s syndrome The events are depicted in Figure 16 with the thickness of the arrows essentially indicating the relative concentration of hormones produced at various steps In Cushing s syndrome it is suggested that there is an increase in ACTH causing a greater stimulation of the adrenal cortex to produce a larger quantity of C_{21} steroids than of C_{19} steroids Thus as a result of the increased quantity of ACTH a greater amount of pregnenolone is produced and in turn converted to an abnormally high amount of progesterone which is efficiently and in high yield converted to hydrocortisone and probably other hydroxylated steroids The important point however is the fact that the hydrocortisone production is significantly higher and is responsible for the symptoms of the disease Why then does this increased amount of hydrocortisone not inhibit the production of ACTH by the anterior pituitary? This question cannot be answered with certainty at this time but the indications are that the anterior

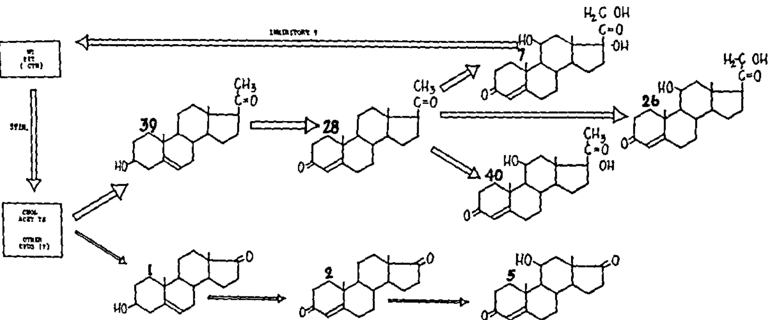


FIGURE 16 Adrenocortical production by the gland in patients with pure Cushing's syndrome Compound names 1 = dehydroepiandrosterone 2 = Δ^4 androstene 3 17 dione 5 = 11β hydroxy Δ^4 androstene-3 17 dione 7 = hydrocortisone 26 = corticosterone 28 = progesterone 39 = Δ^5 pregnene-3β 20 one 40 = Δ^4 pregnene 11β 17α diol 3 20 dione

diminished inhibition of the anterior lobe what is the evidence for that?

Dorfman I do not have it, I suggested a possible explanation However there is some evidence that there is probably an increase in ACTH production

Thorn I should like to modify a statement I made before If a patient with Cushing s syndrome is given ACTH we may over a period of 48 hours obtain levels as high as 70 80 or 90 mg of total 17 hydroxysteroid excretion in the urine We cannot say from our data that the increase in 17 ketosteroid is necessarily greater than we should obtain if the same excess of compound F as measured by the urinary total 17 hydroxysteroid excretion were to be ad ministered Thus I do not believe we have evidence of an increase in 17 ketosteroid fraction by this technique in certain patients which would be consistent with the remarks which Dr Dorfman has made

Gallagher Our results with patients who have classical Cushing s syndrome are in agreement with these results There is a slight increase in the usual 17 ketosteroids but in addition to that there are a considerable number of other compounds present With many of these we have not been able to make a quantitative measure ment the substances are seldom seen in appreciable amounts in normal urine

Long When you say Cushing s syndrome do you mean adrenal hyperplasia with no adrenal or pituitary tumor?

Dorfman Adrenal hyperplasia may or may not occur I really do not know whether all of those people have demonstrable changes in the adrenal but they could still have the Cushing syndrome There is no definable tumor What is seen is only the metabolic effects of high amounts of hydrocortisone

Thorn It is a picture of excess ACTH

Dorfman Yes

Long However these people would have the so called Crooke s changes in the pituitary?

Loeb I think that is a perfectly safe statement

Mason You mentioned a case which we studied some time ago The patient was a boy with Cushing s syndrome who was excreting large amounts of formaldehydogenic steroids and about 80 mg a day of 17 ketosteroids These are the urinary steroids which we succeeded in isolating

In Table IV you will note that there is a rather large amount of dehydroisoandrosterone a small amount of etiocholane one 11

pituitary in Cushing's disease has lost its sensitivity to the inhibitory action of hydrocortisone or the metabolic products which normally produce this effect

A second question that must be answered with respect to Cushing's syndrome is the differentiation between the production of C_{21} and C_{19} steroids Since we have adopted as our primary thesis that these two channels are essentially unrelated it must follow that in Cushing's disease the stimulation of ACTH must result in a relatively lower C_{19} than C_{21} steroid production Thus in the "pure Cushing's syndrome we conceive of the following biochemical defects (*a*) an increment in ACTH production (*b*) a relatively large increase in C_{21} steroid production as compared to the C_{19} steroid production and (*c*) a relative pituitary refractoriness to hydrocortisone

Thorn Do you believe that the C_{19} compounds are actually increased or could the larger quantity be accounted for by the increase in the derivatives coming from the other steroids?

Dorfman They may be accounted for on the basis of the increased amount of metabolites coming from hydrocortisone We are anxious of course to accumulate as many patients as we can to define this as precisely as possible

White Does the *in vitro* stimulation of the gland with ACTH throw any light on this question? If these compounds are arising indirectly then *in vitro* we should expect very little of the lower line compounds to appear but if they are coming directly then possibly ACTH *in vitro* might increase both lines

Thorn The 17 ketosteroids are often fairly normal with a very high F output

Dorfman In such a case I would suggest that the bulk of the 17 ketosteroids would be of the 11 oxygenated etiocholane type

Bauer Would the situation alter if ACTH were given to such patients?

Thorn If ACTH is given to a patient with classical Cushing's syndrome there is a tremendous rise in both the 17 ketosteroids and the 17 hydroxysteroids

One would have to study a patient with Cushing's syndrome who had a high level of 17 ketosteroids not derived from cortisone or hydrocortisone in order to settle this point It would probably require relatively large doses of hydrocortisone to suppress the 17 ketosteroid derivation from non 17 hydroxysteroids

Long You indicated that under these circumstances there is

FIGURE 17 Adrenocortical steroid production by the gland from patients with adrenal cancer Compound names 1 = dehydroepiandrosterone 2 = Δ androstene 3 17 dione 5 = 11β hydroxy Δ^4 androstene 3 17 dione 7 = hydrocortisone 26 = corticosterone 28 = progesterone 39 = Δ pregnene 3β 20 one 40 = Δ pregnene-11β 17α diol 3 20 dione

TABLE IV

Compounds Isolated from the Urine in Cushing's Syndrome With Diabetes During 25 Days

Compound	Weight mg
Dehydroisoandrosterone	130
Etiocholane 3(α) ol 17 one	6
Etiocholane 3(α) 11(β) diol 17 one	33
Pregnane-3(α) 20(α) diol	172
Pregnane-3(α) 17(α) 20(α) triol	35
17 Hydroxycorticosterone	191

oxygenated 17 ketosteroid (etiocholane-3α 11β diol 17 one) considerable pregnanediol and some pregnanetriol

Dorfman Androsterone is missing

Mason It may have been there in small amounts but not very much I am sure

Astwood There was no tumor in this case?

Mason No

Long May I come back to these Crooke s changes because of this factor you have introduced of diminished inhibition of the pituitary? I just consulted my colleague Dr Thorn and our understanding is that when Crooke s changes occur in the pituitary they represent pituitary inhibition since they are produced by giving excessive cortisone to animals

Conn Crooke s changes have been produced in man with therapeutic doses of cortisone (27)

Dorfman We shall continue now with a consideration of the defects that are consistent with the condition of adrenal hyperactivity known as adrenal cancer We are choosing individuals as mentioned earlier who do not reveal the metabolic effects of excessive doses of hydrocortisone and who show only moderate to severe masculinization as a result of increased androgen production The stimulation by the anterior pituitary of the adrenal cancer is open

one Δ pregnene 3β 16α diol 20 one and Δ^5 pregnene 3β 16α 20α triol

That portion of pregnenolone which is converted to progesterone however can be effectively hydroxylated at positions 11 17 and 21 and hydrocortisone is produced usually in sufficient quantity to maintain the individual Other hydroxylated compounds such as corticosterone and desoxycorticosterone (not shown in Figure 17) can be effectively produced Irrespective of the concentration of hydrocortisone the production of the steroids at the adrenocortical level continue at the same increased rate since essentially the adrenal cortex has an autonomy and the action of hydrocortisone on the anterior pituitary is of little consequence Thus the biochemical defects in adrenal cancer appear to be (*a*) the autonomous production of steroids with a preponderance of the formation of C_{19} steroids and (*b*) a relative lack in that enzyme (3β dehydrogenase) which normally converts the Δ -3β hydroxy group to the Δ^4 3 ketone leading to the accumulation of dehydroepiandrosterone and other Δ 3β hydroxy steroids

Thorn The theory is sound regarding ACTH but one must keep this fact in mind Patients with cancer of the adrenal and with inhibition of remaining adrenal cortical tissue may respond to ACTH if it is given in large doses over a continued period of time Under these circumstances one might anticipate a slow gradual rise in the secretion of steroid from the atrophic adrenocortical tissue in contrast to the very rapid and large rise which one would anticipate in a patient with bilateral hyperplasia of the adrenals receiving a similar quantity of ACTH Thus in cancer of the adrenal one could not exclude a normal adrenocortical response if adequate ACTH were administered

Ralli If the malignant tumor is removed from these patients — and presumably it attacks only one adrenal — does the other adrenal function normally?

Dorfman In most of the patients we have studied the steroid excretion will stay down for a few months after removal of the primary tumor

Conn I should like to ask Dr Dorfman what kinds of adrenal cancer he is referring to One adrenal cancer may produce a picture that looks like Cushing's syndrome others may be masculinizing or feminizing From what kinds of cancer patients are these data derived?

Dorfman This would be a condition where the patient shows a minimum of or no metabolic changes attributable to high concen

to question It is most likely that although ACTH may be produced and circulating it is of little consequence with respect to the production of steroids by this cancerous adrenal In other words we are saying that the adrenal bearing this cancer has now achieved a certain autonomy of hormone production

The cancerous adrenal tissue produces increased amounts of both C_{21} and C_{19} steroids (Figure 17) with perhaps the usual pattern showing the preponderance of C_{19} steroids This point will not be decided with certainty until adequate methods are available for precise quantitation of total C_{19} and C_{21} steroids Under these conditions there could be and usually is a large increase in the production of dehydroepiandrosterone as one of the first products from either the cholesterol or acetate It will also be noted that the transformation of dehydroepiandrosterone to Δ^4 androstene-3 17 dione is represented by an arrow thinner than that indicating the production of dehydroepiandrosterone An important factor in the disease seems to be that although the tissue can produce relatively enormous amounts of dehydroepiandrosterone there appears to be a relative lack of the 3β dehydrogenase which oxidizes the Δ -3β hydroxy group to the Δ^4 3 ketone Thus only a portion of the dehydroepiandrosterone can be converted to the Ring A oxidized form That part which is converted to Δ^4 androstene 3 17 dione however can be 11β hydroxylated and on the basis of our preliminary analysis of individual components of the C_{19} series it is evident that there is a preponderant increase in urinary products derivable from Δ^4 androstene 3 17 dione and 11β hydroxy Δ^4 androstene 3 17 dione However the accumulation of dehydroepiandrosterone is the most striking part of the urinary steroid pattern Since the 11β hydroxylase does not efficiently operate on compounds having the Δ^5 3β hydroxy group the presence of the 11β hydroxy derivative of dehydroepiandrosterone is probably formed only in minute amounts and has as yet not been isolated Instead some of the dehydroepiandrosterone appears to be efficiently converted to various other derivatives such as *Δ androstene 3β 17β* diol and Δ androstene 3β 16α 17β triol

With regard to the C_{21} steroids it is found that a relative increase in pregnenolone would be consistent with our thesis and that a good portion but not all of this substance would be converted to progesterone The accumulation again of the Δ -3β hydroxy group compound is seen from the fact that in adrenal cancer there is excreted an increased quantity of the following Δ 3β hydroxy compounds Δ^5 pregnene 3β 20α diol Δ pregnene-3β 17α diol 20

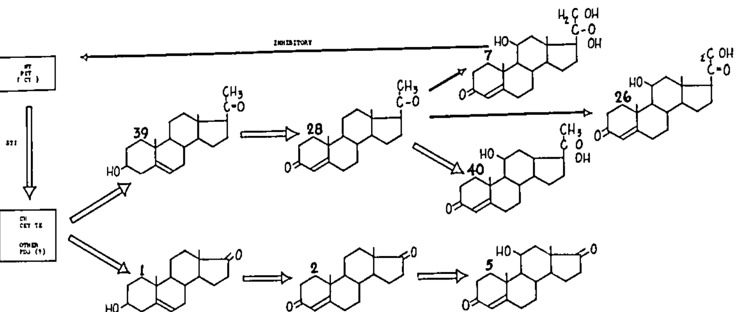


Figure 19 Adrenocortical steroid production by patients with the adrenogenital syndrome Compound names 1 = dehydroepiandrosterone 2 = Δ^4 androstene-3 17 dione 5 = 11β hydroxy Δ^4 androstene 3 17 dione 7 = hydrocortisone 26 = corticosterone 28 = progesterone 39 = Δ^5 pregnene 3β 20 one 40 = Δ^4 pregnene-11β 17α diol 3 20 dione

trations of hydrocortisone certainly not the massive disease that is seen in Cushing's syndrome

Ingle Is there evidence that metastases of adrenal tumors can secrete hormones?

Dorfman I am fairly sure they do because when metastases are present the steroid production increases significantly

Long There are a good many examples of that from the cases of the Mayo Clinic collected by Dr E J Keppler where the tumor was removed and later metastases in the liver appeared with a recurrence of Cushing's syndrome

Dorfman I should like to consider the biochemical defects in the adrenogenital syndrome patient These events are depicted in Figure 18 in which it will be noticed that the representation includes the fact that increased amounts of ACTH are produced by the anterior pituitary

Experimental proof for this fact has been made available by the direct analysis of the blood ACTH in children by Sydnor (28) Thus increased ACTH production by the anterior pituitary results in an increased formation of both C_{19} and C_{21} steroids In other words both dehydroepiandrosterone and pregnenolone are produced in increased quantities and each of these substances is efficiently transformed to the Δ^4 3 keto compound In the C_{21} series we should therefore feature an efficient production of progesterone which can now be hydroxylated In the C_{19} series an increase in the amount of Δ^4 androstene 3 17 dione is present and this compound in turn is hydroxylated at position 11 to form increased amounts of 11β hydroxy Δ^4 androstene 3 17 dione The fact that this 11 hydroxylated androgen is present in increased amounts (29) is evident from the fact that in patients of this type there is an enormous increase in 11 oxygenated androsterone Thus at this point we can say that without question there is not a decreased amount of 11β hydroxylase in these adrenals but rather that the concentration of this particular enzyme is actually increased in total and perhaps in enzyme unitage per gram of tissue

Coming back to our consideration of the increased production of progesterone we find from the evidence of urinary studies that both pregnane 3α,20α diol and pregnane 3α 17α 20α triol are increased enormously in this syndrome The pregnane 3α 20α diol would be a reflection of the increased progesterone production but the presence of increased amounts of pregnane-3α 17α 20α triol indicates to us that 17 hydroxylation has efficiently taken place and in fact at an increased rate Thus we may say that of the three

Dorfman Also although not shown in Figure 18 there should be a relative lack of desoxycorticosterone because that too would require efficient 21 hydroxylation

Pincus Dr Thorn I believe you once supplied us with urine from a patient getting large doses of adrenosterone and as I remember there was a definite inhibition there of pituitary activity Is that right?

Dorfman When large doses of adrenosterone were administered we had somewhat of a decrease in the products normally derived from Δ^4 androstene-3 17 dione

Long However if the patient has large amounts of corticosterone why should he run into salt deficiency?

Dorfman He cannot have corticosterone in large amounts because the 21 hydroxylation reaction is deficient This would cause a relative deficiency in cortisone hydrocortisone desoxycorticosterone and possibly electrocortin

White There is the example of the young patient with adrenal hyperplasia who shows an inability to retain salt and in whom the administration of DCA is quite ineffective because there appears to be in this instance not only an inability to make DCA but the production of something which may be anti DCA Does such an individual respond quite well to exogenous DCA?

Conn They respond to DCA but the administration of ACTH may intensify the salt loss

Thorn Although I think one might predict Addison s disease on the basis of refractory renal tubules without evidence of over all kidney damage we have never observed the patient with intact renal function by other measurements who did not respond to desoxycorticosterone with sodium chloride retention It is only in total renal failure that we have detected the rare cases of salt losing nephritis which are completely refractory to desoxycorticosterone

Conn I think there is one other point which fits in very well and that is the occasional case of adrenogenital syndrome that develops hypoglycemia We have followed one such case Each time the patient had an infection hypoglycemia resulted from that stress I believe Wilkins (30 31) has two cases showing the same phenomenon

Dorfman If these ideas are correct we should be able to find relative differences in concentration of certain enzymes such as those responsible for 11β 17α and 2 hydroxylation as well as the enzyme which converts the Δ 3β hydroxyl to the Δ^4 3 ketone

hydroxylation enzymes that are necessary to produce hydrocortisone from progesterone two are actually increased these being the 11β and 17α hydroxylase However there is a proportionately decreased amount of 21 hydroxylase as evidenced by the fact that the amounts of hydrocortisone and corticosterone produced are relatively low Thus it appears that since the hydrocortisone con tent is kept relatively low, insufficient hydrocortisone would be available to inhibit efficiently, the anterior pituitary the latter gland would therefore continue to produce its increased amount of ACTH Thus we may say that there is one cardinal defect in this system that is a relative lack of 21 hydroxylase, which causes an insufficient quantity of hydrocortisone to be produced which in turn leads to increased ACTH production and finally an increased production of steroids by the adrenal As has already been men tioned when we discussed the normal steroidogenesis by the adrenal of particular importance in this scheme is the fact that the androgen production is dependent upon the relationship between the quantity of hydrocortisone produced and the amount of ACTH produced Thus in this syndrome the lack of 21 hydroxylase leads to an increased production of androgens which in turn are re sponsible for the clinical syndrome Recent clinical work by Wilkins and co workers (30) indicates that the symptoms of the patients suffering from this disease may be promptly corrected by the administration of hydrocortisone or cortisone This is consistent with the scheme presented since the additional amounts of hydro cortisone would effectively cause inhibition of the anterior pituitary and the direct result would be decreased production of the andro genic hormones which are the offending steroids in the adreno genital syndrome

Thorn Would you say that the large arrow in Figure 18 which indicates coming from the pituitary may not necessarily be the initiating factor but rather a secondary factor and that the increased *secretion of ACTH is the result of the process rather than a cause of the syndrome?*

Dorfman I think that is probably so

Thorn We have had one patient with this syndrome who showed a good eosinophil fall with ACTH which we interpreted as repre senting the secretion of some 17 hydroxysteroids but who subse quently *died of salt retaining hormone deficiency* We are impressed that salt deficiency *is one of the most characteristic clinical aspects* of patients with this syndrome

some people fortunately produce enough enzymes to balance the effect of increased ACTH production

Dorfman That is right it may be a question of enzymes In other words what I say is that people probably vary in the amount of ACTH they produce but if there are proportionate amounts of enzymes the hydrocortisone production is adequate to prevent the ACTH production from increasing to the point of producing excessive amounts of androgens

Loeb I believe what Jailer has been hoping for is that someone would find a very large adrenal in one of these people which might be removed and perfused

Dorfman We have perfused some of these glands from patients with the adrenogenital syndrome but have learned little

Ralli If we were to isolate or identify the principal enzymes involved is there any reason to think that the information might be a clue to the situation?

Dorfman Are you suggesting that enzymes should be administered?

Ralli Yes

Pincus I believe it may not be the enzyme at all but some necessary cofactor of the enzyme For example according to Bush (32) the rat adrenal produces mostly corticosterone which is a 17 desoxycorticosteroid This has been confirmed by other people Nevertheless Heard (33) and Hofman and Davison (34) have both incubated the rat adrenal *in vitro* and obtained 17 oxygenated steroid from it So the *in vitro* picture is not the same as the *in vivo* picture

What is the explanation? I suggest that the enzyme system is there all right but that *in vivo* there is either an inhibitor preventing it from acting or a cofactor which is absorbed so fast by the adrenal tissue that it cannot be used whereas in the *in vitro* experiment there is either a storage of the cofactor or the inhibitor is destroyed I think it is probably a cofactor

Ralli A cofactor which might be the coenzyme

Pincus Another reason why I think it is a cofactor particularly in this adrenogenital syndrome is that it is corrected by giving steroid and testing the pituitary inhibition

Dorfman Yes we give them the product which they cannot make to bridge that step

Pincus I believe that Wilkins (30) finds that the recovery is then complete or practically complete

Thorn I wonder whether we should postulate an increase in the total metabolic pool of steroids within the adrenal as a part of this syndrome It would seem that many of the cases that are seen might be explained on a shift in the distribution of the steroid rather than in alteration of the total quantity

Conn Assuming the validity of Dr Dorfman s thesis should we not regard the adrenal hyperplasia as compensatory, the primary defect being of adrenal origin, and increased ACTH production representing the normal response of the normal pituitary to such circumstances? That is the initial defect is one which does not allow enough 21 hydroxylation serum F falls ACTH increases in an effort to raise serum F and secondary hyperplasia occurs whether or not the functional compensation has been accomplished This would mean then from a therapeutic point of view that if some thing other than F or an F like compound should be discovered which could inhibit ACTH activity or production it would be the wrong thing to use in these cases since it would precipitate adreno cortical insufficiency

Dorfman An interesting point is that there may be a number of people leading a normal life with a relatively high level of ACTH activity but they are fortunate in having the compensation of ade quate enzymes to carry on with so that ACTH level does not get out of bounds consequently the production of androgens does not become high enough to produce clinical symptoms

Conn That again brings up the matter which Dr Thorn raised earlier regarding his patient with high 17 ketosteroid and low 17 hydroxycorticoid excretions

Thorn Which compounds have you studied in the first step to demonstrate the failure of oxidation at carbon 21?

Loeb Dr J W Jailer* has given 17 hydroxy progesterone to these people and has found no increase in 11 oxysteroids which is what one would expect

Gallagher Even if a very large amount were given I should suspect a very tiny amount would go through the adrenal and the rest would be metabolized Only a small portion would be sub jected to hydroxylation at C 11

Ralli Could you look at this from another point of view? There may be some interference in the enzyme system and this may be the initial step in disordering the mechanism and perhaps stimulat ing an oversecretion of the hormone In other words you say that

*Personal communication

correctly that was hyalinization of the pituitary beta cells Is that correct?

Dorfman Yes

Young That would indicate a dysfunction of the secretory function of ACTH would it not?

Thorn Yes that is correct It is probable that the Crooke s changes may represent the long continued effect of excessive adrenal steroid level and are not the initiating cause of the Cushing s syndrome

Astwood It may be like Graves disease where there is no evidence for an increased production of thyrotropin it is as though something else were wrong with the thyroid and the normal amount of thyrotropin would make it work too much Perhaps that is the way it is in Cushing s syndrome

Thorn The best example of that is to give a small increment of ACTH to a patient with Cushing s disease and measure what happens or give a standard dose of ACTH to a normal individual whose adrenals have been activated a bit The physiological effect of the hormone is intensified tremendously

Ralli Dr Astwood are you suggesting that the cellular situation of the adrenocortical cells would be the determining factor as to the direction in which the reaction would go when the pituitary stimulus came through?

Astwood No I was thinking that in the primary overactivity of an endocrine gland such as the adrenal in Cushing s syndrome and the thyroid in Graves disease the fundamental defect is not excess of the tropic hormone but something else at present unknown

Ralli It might be some enzymatic change within the cell?

Astwood It is probably something more complicated than that because the entire gland is involved

Ralli If the rat is any index of what may happen a deficiency of pantothenic acid will seriously affect the integrity of the adrenal cells and under these circumstances if the animal is stressed the adrenal enlarges

Thorn In regard to secretory activity it is quite possible that if we put out the ordinary amount of ACTH over a 24 hour period rather than over the 12 hour period as our data suggest we might all be suffering from Cushing s syndrome

Pincus With a chronic insomnia?

Thorn It is possible in certain instances that patients with thyrotoxicosis and Cushing s syndrome are merely individuals who

Thorn As a matter for the record has anyone ever demonstrated by direct measurement that cortisone inhibits ACTH in the adrenogenital syndrome?

Dorfman If we add the fact that we obtain a decrease in the steroid production by cortisone or hydrocortisone administration to what has already been reported in the literature that there is initially an increase of ACTH in the blood I think it gives a good working point

Thorn However an increase in blood level could be explained by an alteration in the rate of destruction or fixation of the hormone as well as on the basis of increased secretion One could assume that the adrenal cortex itself might be very susceptible to cortisone action and certainly these experiments do not exclude a direct action of cortisone on the adrenal cortex

Pincus You may be perfectly right We can now say more definitely that if we perfuse the steer adrenal we obtain very little F but a good deal of B This difference between steer and cow adrenals may be the result of castration It may be that the gonad hormones have a direct rather than an indirect effect

Thorn It is entirely possible that the adrenal cortex itself may be very sensitive to high or low levels of hydrocortisone

Dorfman Was Jailer s work on a hypophysectomized person with adrenogenital syndrome?

Loeb No it was adrenal carcinoma

Thorn I do not think it changes your theory

Ingle Has Wilkins given ACTH to these patients and shown that the adrenogenital syndrome is aggravated?

Dorfman I believe that this has been done

Young Mason and Morris (35) did that They tested a girl with congenital adrenal hyperplasia who ultimately died of adrenal insufficiency in spite of cortisone treatment What evidence is there to preclude the possibility that the enzyme dysfunction is not in the adrenal but in the systems wherever they are in the body that dispose of F and B so that they are being degraded more rapidly than normally and therefore not available for ordinary purposes? If F and B were lacking the pituitary would not be inhibited ACTH would be produced in excess and C_{19} steroids such as dehydroepiandrosterone would appear

Ralli Does the cholesterol level in the blood change in patients with this disease?

Dorfman I do not know

Young May I ask about the Crooke changes? If I remember

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Ralli Does the cholesterol level in the blood change in patients with this disease?

Dorfman I do not know

Young May I ask about the Crooke changes? If I remember

Dorfman The bulk of the androgenicity in the adrenogenital syndrome comes from the C_{19} 11 oxygenated compounds I believe that 11β hydroxy Δ^4 androstene-3 17 dione together with some Δ^4 androstene-3 17 dione are the responsible compounds

White How high is the androgenic activity of 11 = beta hydroxyandrostenedione?

Dorfman We cannot assess the androgenic activity in humans We have only rough measures at the moment in chicks probably of the order of one third as active as Δ^4 androstene-3 17 dione

Gallagher It is androgenic?

Dorfman Yes

Gallagher Salamon and Dobriner (36) isolated 11β hydroxy Δ^4 androstene-3 17 dione from the urine of patients treated intensively with ACTH It seems reasonable to believe that ACTH exaggerates the normal adrenal secretion and thus their finding would support the view that 11β hydroxy Δ^4 androstene-3 17 dione is normally secreted in small amounts by the adrenal gland This compound therefore may be one of the adrenal androgens Some of the metabolites found in the urine during ACTH stimulation may well be derived from this precursor

Thorn In so far as excretion products can be interpreted as reflecting original androgen activity of the steroid we know that removal of the adrenal glands in a castrate male gives a marked reduction in biological androgen activity These studies have been carried out by Dr Paul Munson of the Harvard School of Dental Medicine (37) In other words the material in the urine is biologically androgenic as measured by a technique which one accepts as measuring androgenic activity

White Was this assay in the chick or the rat?

Long In the chick If we restore the 17 ketosteroid level with cortisone or compound F in the patient without adrenals the androgenic activity of the 17 ketosteroid fraction in the urine is entirely different than it is in the individual who let us say had a daily excretion 5 mg of 17 ketosteroid from his own gland

Dorfman It is lower?

Long Much lower in fact it is almost nonexistent until the amount of cortisone is increased to 100 mg

White Delta 4 androstenedione is a potent androgen in the chick It is weakly androgenic in man it has been given to women with no striking development of masculinization I am still curious to know what the biological activity in urine is when assayed in the chick in relation to the masculinization which we see in man

have *a normal total 24 hour* secretion of *TSH* (thyroid stimulating hormone) or ACTH, with no diurnal variation

Ingle *Dr* Dorfman *do you believe that the normal* adrenal cortex *secretes* physiologically significant amounts of androgen?

Dorfman Yes *I do* *I* believe the adrenal cortex directly produces C_{19} compounds Dehydroepiandrosterone has not been isolated *from the tissue but on the basis of all the evidence* it *appears to* be secreted by the gland Δ^4 Androstene 3 17 dione is also pro *duced by the gland together with* 11β hydroxy Δ^4 androstene-3 17 dione and both are secreted into the blood stream Analysis of *adrenal perfusates adrenal venous blood* and metabolic considera tions all point to an actual production and secretion of the C_{19} androgens

Loeb For years I have been impressed by *the lack of overt and significant clinical manifestations of any loss of androgenic material in* the Addisonian patient *I should like to* ask what the experience is in totally *adrenalectomized individuals so long as one provides* say salt and water hormone and cortisone what evidence is there of a significant physiological deficit in androgenic steroids?

Long There have been several series of experiments in which the testes have been removed from an animal let *us* say *a male* rat *or mouse* In that case there is marked atrophy of the prostate and seminal vesicles The administration of ACTH in any amount one wishes to give brings about no repair of the prostate or seminal vesicle I believe that your laboratory did some of these experi ments Dr Li

Li Yes We recently used hypophysectomized castrated male rats to investigate the influence of ACTH on the secondary sex organs *ACTH* was administered with either gelatin or beeswax as injection *media* producing an adrenal 8 to 10 times *the* size of the controls There is no evidence whatsoever of androgen secretion from the adrenal

White We first used the word androgen as a blanket term for C_{19} compounds but now we are *shifting our* interest to bio logical activity

Loeb The question was about biological activity

Li Dr Loeb is right *we were measuring the androgenic activity*

White In that *connection* I was going to ask whether the adrenal does not produce testosterone I gather that *there* is *no* evidence of where the androgenicity that one sees in the adreno genital syndrome stems from

tion were to diminish for any reason in conjunction with adrenocortical insufficiency the reduced estrogen secreted by the ovary and the reduced androgen secreted by the adrenal might possibly balance each other to some extent with respect to physiological activity The evidence however is meager for this point with one exception and that is in patients with genetic predisposition for hirsutism Under these circumstances ACTH treatment in young women can result in a marked change in hair growth This may become a serious social problem for these patients whereas in other individuals who do not have this inherent capacity of increased hirsutism ACTH treatment may be tolerated quite well The same comments are applicable with respect to the development of acne

Dorfman How high were these doses of Δ^4 androstene 3 17 dione that were administered to women in whom you did not see the masculinization?

White Two hundred milligrams a day There were no data as to the extent to which it was absorbed

Gallagher From isotopic experiments we know the compound is readily absorbed It surprises me to hear that it is not androgenic Judged by the capons comb response the compound is a good androgen

Ingle Dr Dorfman do you think that the adrenals and gonads are the only tissues of the human that can secrete steroid?

Dorfman Even after the removal of both the gonads and adrenals of male and female monkeys we still found small amounts of androgens by a chick comb test

Ingle In your studies on monkeys did you exclude food as a possible source?

Dorfman No extensive studies on food were done

Mason Didn t you maintain those animals on cortical extract at one time?

Dorfman During the critical test periods many of them were maintained only with salt

Ralli We repeated some of the work that Dr Pitts (42) reported at these meetings in 1951 You remember that he observed in the adrenalectomized rat that the capacity to handle an acid load was considerably impaired We were interested in seeing whether this test would serve as a measure of the effect of certain nutritional substances in adrenalectomized rats Male and female intact and adrenalectomized rats were studied In the adrenalectomized rats an acid load was given five to seven days following the removal of the adrenal and the ammonia and titratable acid excretion were

and whether we have any indication of what compound is responsible for the masculinization?

Dorfman It would be surprising if there were not a dose that would produce a true androgenic effect in man but the relative amount of Δ^4 androstene 3 17 dione that would be needed might be enormous

Long Dr Dorfman has ACTH been given to test this point as to whether sufficient androgen will be produced to correct the hypogonadism?

Dorfman I do not know of any studies of that sort

Long From the evidence before us it would appear that the rodent adrenal is different from the human but here would be an opportunity to prove it, if people were available

Dorfman In 1936 Moon (38) reported the fact that with crude ACTH preparations in a gonadectomized animal he was able to obtain androgenic stimulation of the prostate

Li With the crude extract which Dr Moon used we did obtain some suggestive stimulation in castrated hypophysectomized male rats However highly purified ACTH preparations cause greatly hypertrophied adrenals but no increase in ventral prostate weight The only effect in the latter direction is produced with the crude extract We have been wondering whether this might be another pituitary factor

Astwood Dr W P Van der Laan (39) has studied that also, and has shown a direct effect of pituitary extracts on the male genital tract in the absence of testes and adrenals

Pincus We may extract 11 beta hydroxyandrostenedione from adrenal vein blood (40) and Dr Eli Romanoff (41) has isolated it from human vein blood However as far as I know it has not yet been found in the rat blood and we do not thus far understand its biological effects

Dorfman We have tested it on the mammalian indicators have we not?

Pincus It is active but the question is how active is it in the human? Dr Thorn do you have any data on adrenosterone in terms of androgenic effect?

Thorn We had a small quantity available which we administered over a short period of time In a matter of a few days one would not expect to observe any androgenic effect Returning to Dr Loeb s question we should keep in mind that the lack of androgen in a female patient with Addison s disease would have to be considered with respect to the level of ovarian function Thus if ovarian func

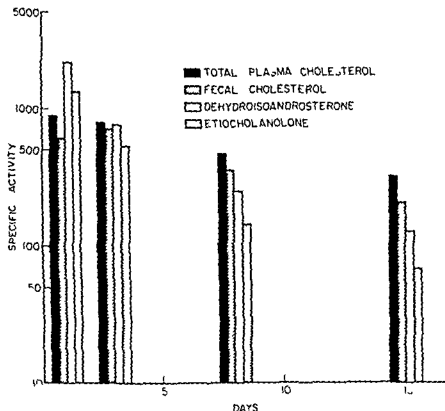


FIGURE 19 The comparison of the specific activity of cholesterol and urinary steroids after a single dose of acetate 1 C in a patient with an adrenal tumor Reprinted by permission from Gallagher T F Biochemical problems of the steroid hormones *Chemical Specificity in Biological Interactions* No 3 Chapt. V New York Academic Press 1954 (pp 50 64)

androsterone and etiocholane obtained from a single day s collection from this patient was insufficient to purify without the addition of carrier material The procedure we adopted with these two substances was similar to that described in an earlier report at the Laurentian Hormone Conference (4) The alpha ketosteroids were separated by chromatography the infrared spectra of the separated fractions were examined and what was believed to be essentially pure androsterone and etiocholane were separately sublimed in high vacuum the amount present was determined from the weight of the sublimate and the quantitative Zimmermann reaction A known amount of carrier was then added and the products were recrystallized to radiochemical purity It was not

determined over a four hour period An interesting difference was noted between the female and male adrenalectomized rats the former had a greater capacity to handle the acid load than did the latter The female adrenalectomized rats excreted about 28 mEq of ammonia per hour per 100 gm of weight compared to 22 mEq for the male adrenalectomized rats The female animals excreted about 3 5 mEq of titratable acid as compared to 2 5 mEq for the males These values may be compared to the average excretion of ammonia of 56 mEq per hour per 100 gm of weight for the intact female and 48 mEq for the intact males The average intact female excreted about 9 mEq per hour of titratable acid compared to 7 5 mEq for the intact males After adrenalectomy plus gonadectomy the excretion of ammonia by the female animals was greater than that of the males (7 5 mEq per hour for the females 4 9 mEq for the males) There is no significant difference in the excretion of titratable acid after adrenalectomy plus gonadectomy (3 5 mEq per hour per 100 gm of weight for the females and 3 2 mEq for the males)

Gallagher I have a figure to present which may be of interest it deals with a portion of Dr Dorfman s diagram Figure 19 is a study of a man with a massive adrenal tumor who excreted very large amounts of dehydroisoandrosterone Enough was found in a single day s excretion of urine to isolate and carefully purify this and other substances At the same time we studied the total plasma and fecal cholesterol We compared the relative specific activities of these products a short time after the feeding of a single dose of carbon labeled acetate

On the first day the specific activity of the two metabolites was somewhat higher than either the plasma or the fecal cholesterol This can be interpreted as indicative of possible independent synthesis on the first day as well as conversion of cholesterol into these substances As the experiment progressed – and I draw attention to the fifteenth day – the specific activity of the steroid metabolites was somewhat lower than the specific activity of the plasma cholesterol suggesting perhaps another path of synthesis than that through cholesterol The experiment I believe indicates that both cholesterol and acetate may serve as precursors for these urinary steroid metabolites

Dorfman Dr Gallagher where is the androsterone in the figure?

Gallagher The specific activity of the androsterone was nearly identical with that of the etiocholane It was not included on the figure in order to simplify the presentation The amount of

an indication that an adrenal tumor has the ability to synthesize cholesterol of higher specific activity than that which is circulating in the plasma at the same time We do not have many experiments of that type because such tumors are fortunately rare When we are concerned about individual organ pools as well as whole body pools we really are on very perilous ground For that reason I think I should prefer not to speculate I would repeat however that the results demonstrate that these steroid hormone metabolites can be synthesized from cholesterol and suggest that there is an independent synthesis from another precursor possibly acetate

Dorfman We did a similar study as you know in which we administered acetate 1 C^{14} to an adrenal cancer patient who was excreting something of the order of 380 mg of 17 ketosteroids per day and it is interesting that the specific activity of the dehydroepiandrosterone the etiocholane 3α ol 17 one and the androsterone isolated from the urine were within 15 per cent of each other and the specific activities of androsterone and etiocholane isolated in this particular case were equal

Pincus I think Dr David Stone and Dr Oscar Hechter have more direct evidence which should appear shortly in which they perfused C^{14} labeled acetate and cholesterol in the absence and in the presence of ACTH There is a 17 fold increase in total radio activity of the recovered F and B from cholesterol after ACTH and only a 2 5 fold increase after acetate That would suggest very clearly a pathway independent of ACTH

Ralli Does the concentration of cholesterol in the adrenal bear any relation to its ability to produce a given quantity of these steroids?

Pincus That was a question which we were unable to answer with the bovine adrenals because the concentration was so low that we could not relate it

Long In rats that are seriously burned or subjected to a severe hemorrhage the concentration of adrenal cholesterol falls 24 hours later to approximately 1 0 per cent or less That would be the time we should expect the adrenocortical hormone content in the urine to have risen Therefore we may argue that the human adrenal at that time would be very low in lipoid as well as in cholesterol I expect that it is

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necessary to add carrier material to the dehydroisoandrosterone and for this reason the specific activity of that compound was the most accurate of the three metabolites examined At this stage of the disease large amounts of androsterone and etiocholane were not present in the urine The specific activity of the androsterone and the etiocholane were in each period lower than the specific activity of the dehydroisoandrosterone

Dorfman The etiocholane appears not to be arising solely from the dehydroepiandrosterone in this case, but rather part arises from the 17 hydroxy 11 desoxycorticosterone and/or 17 hydroxyprogesterone Is the difference in specific activity between dehydroepiandrosterone and etiocholane 3α ol 17 one significant?

Gallagher Very probably although one cannot say so very emphatically It seems significant that the specific activity was lower *in each interval examined but particularly toward the end of the* experiment the errors in counting these low specific activities were appreciable

Bloch These differences are very great

Gallagher The difference on the first day between the plasma cholesterol and the isolated urinary steroids is significant However one must remember that the plasma cholesterol specific activity represents a point in the day whereas the urinary specific activity represents the composite of the whole 24 hours

Bloch I doubt whether this is an argument in favor of independent synthesis You would by the same token have to conclude that the fecal cholesterol was synthesized independently from the plasma cholesterol

Gallagher We know that the fecal cholesterol is continually diluted by the dietary cholesterol The specific activities indicate a precursor product relationship since the only source of radioactivity is in the administered acetate

Bloch How do you explain the differences between dehydroepiandrosterone and etiocholane? Would you have to postulate three independent pathways one for cholesterol another for the C_{21} compounds and a third for the C_{19} steroids?

Dorfman We do not like the idea

Bloch I think it probably can be explained on the basis of different pools

Gallagher This is one of the knotty problems in the interpretation of these results We know little about the pool from which these metabolites are derived we do not know the specific activity of the adrenal tumor cholesterol From other experiments we have

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ACTH — A SINGLE SUBSTANCE OR A MIXTURE OF HORMONES*

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IN 1951 WE advanced the view that there might be a separate factor acting on the adrenal gland other than what is ordinarily called ACTH (1) However before I discuss our own researches I should like to touch on the lines of thought which seem to indicate that ACTH may in fact contain more than one separable factor The first suggestion of this sort that I am aware of was in 1951 when Talbot and his colleagues (2) observed that the administration of ACTH causes a simultaneous increase in both urinary 11 17 oxycorticosteroids and 17 ketosteroids in humans but in stress presumably under the influence of endogenous ACTH from the subject s own pituitary gland the 17 ketosteroid excretion diminishes while that of the 11 17 oxycorticosteroids rises Therefore Talbot and his colleagues inferred that the human pituitary gland secretes two ACTH s one concerned with 11 17 corticosteroid secretion and the other with 17 ketosteroid formation

First a word about terminology I like to use the term ACTH as one which covers any substance present in the pituitary gland that influences the adrenal gland I think there may be some controversy on that topic and the question of corticotropin or corticotrophin is a matter that sometimes comes up for consideration I shall use the term ACTH" as a general one to cover any pituitary substance acting on the adrenal gland in any respect and whether or not it brings about increased nourishment as occurs in the case of corticotrophin

It is obvious that there are many alternative interpretations to these experimental findings some of which were touched upon earlier but it is interesting in view of the more recent experiments of Winter Hollings and Stebbins (3) to find that simultaneous

* The research on which the following discussion is based was carried out in great part by Mr M P Stack Dunne and Mr H B F Dixon. On the histological aspect of the work we are grateful for the collaboration of Dr D B Cater

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for the ascorbic acid reducing test and melanophore expanding effects However this does not preclude the possibility that the substance effective in the Sayers test for ascorbic acid reducing activity does possess a small intrinsic melanophore expanding activity The loss of this on heat treatment might be relatively small and might be obscured by potentiation of the type I have just mentioned An overlap of properties such as might occur between ACTH active in the Sayers test and intermedin would perhaps be comparable with that already observed by du Vigneaud and his colleagues (9) to hold for vasopressin which possesses intrinsic oxytocic action in addition to its pressor and antidiuretic activities in contrast to purified oxytocin which does not appear to possess pressor or antidiuretic activity

The possibility of an overlap of properties of that sort between various factors can be an extremely complicating one in the efforts to separate hormones and it is not one that tends to be observed at the early stages of the work But it is one that we cannot neglect at the present time

The possibility that pigmentation in Addison s disease and under other conditions might be related to these activities is something that I am not particularly competent to discuss but it is of some interest if ACTH has intrinsic action of the melanophore expanding type and is also concerned with the development of melanin pigmentation

Sulman (10) has suggested that ACTH is a complex consisting of three factors one active in the Sayers test the second in an adrenal weight increasing test and the third a substance which he calls the chromatophore hormone (intermedin) However I think the evidence available suggests that the ascorbic acid reducing activity and the adrenal weight increasing activity of the alkali treated intermedin are not very substantial

More recently Johnsson and Hogberg (11) in agreement with some earlier observations of Hungerford Reinhardt and Li (12) found that alkali heated ACTH does possess some eosinophil reducing activity Johnsson and Hogberg state that alkali heated ACTH also induces an increased urinary excretion of potassium and of dehydroepiandrosterone in the human being while leaving the total 17 ketosteroid excretion unchanged They make no comment about the implications of this finding but they leave open the possibility that after alkali treatment there is some physiologically significant action other than that of melanophore expanding activity

administration of androgen, methylandrostenediol, and cortisone can induce repair of the adrenal glands in hypophysectomized rats although in these experiments neither steroid alone produced such complete repair Some comparable observations have been made by Zizine (4)

According to Winter and his colleagues the adrenal cortices of hypophysectomized rats given androgen methylandrostenediol and cortisone appear to be essentially normal as far as histology is concerned Thus one may consider the possibility that the extent of adrenal repair induced by ACTH may depend in part upon the rate at which the *secretion of androgen is stimulated* as compared with the stimulation and secretion of adrenal steroids of the C 1 type Whether such variations if they do occur would be associated with intrinsic variabilities in the enzyme pattern of the adrenal cortex or with the presence of different fractions in ACTH one of which is particularly potent in stimulating the secretion of androgens *of the adrenal cortex is a problem which I do not* wish to discuss at the present time I feel that perhaps the simplest interpretation is that there is variability in the adrenal cortex and that there is no clear evidence from these observations that there is more than one ACTH I am not inclined to accept the view that the endogenous ACTH in man is different from that which is administered as con ventional ACTH

The second matter which I wish to touch on is the possible relationship of ACTH and intermedin with which we ourselves have not been directly concerned in recent years We were interested in the question of intermedin a good many years ago and Dr Collip (5) first made suggestions about the relationship of inter medin with a metabolism stimulating pituitary factor but the recent suggestions by Johnsson and Hogberg (6) and also by Sulman (7 8) that there is a relationship between ACTH and intermedin certainly comes under the scope of the present discussion

I think it is to be accepted that the purest preparations of ACTH so far obtained do possess some melanophore expanding activity However it is also true that when such preparations are heated in 0 1 normal NaOH for from 5 to 10 minutes at 100 C there is an almost complete loss of ascorbic acid reducing activity with a small loss of melanophore expanding activity Furthermore in some crude preparations of pituitary extracts one finds that heating in alkali appears to potentiate the melanophore expanding action

I think it is clear from the experiments on heating in NaOH that there can be no simple *identity between active structures responsible*

pH 8 5 in the cold room and we usually Seitz filter the extracts and keep them in a sterile condition at pH 8 to 8 5 This extract contains substantial growth promoting activity Tested in the hypophysectomized rat it contains thyrotropic and gonadotropic activity but in the Sayers test it is virtually devoid of action We find in fact calculating from the average of a large number of estimates made on material of this sort that about 70 gm of the material in this extract contains one International unit of ACTH as measured in the Sayers test and I do not think that can be regarded as significant that effect can be obtained only by giving very large doses intravenously in the Sayers test

Ralli May I ask if you assayed this in hypophysectomized albino rats?

Young Yes for the most part however we did not always use albino rats

Ralli We found that in the two weeks following hypophysectomy in young black rats (14) there was an increased deposition of pigment in the hair apparatus of the animals as the adrenal atrophied This was similar but not as intense as that observed when we removed the adrenals from black rats (15)

Young Was this in entirely black rats?

Ralli Yes it is easier to observe in black rats

Young The two types we have used are Norwegian and hooded rats black and white or all white We have not noticed any changes in the hair

Ralli The pigment changes are not discernible in the black and white or all white rats but a change should be observed in the hair growth (16 17)

Young We certainly have not looked for that particular point As I say it is not at all clear as to why this crude alkaline extract is so deficient in activity in the Sayers test because one can show by extraction with acid acetone for instance that the ox anterior pituitary tissue that we have used has a reasonable amount of ACTH activity as measured in the Sayers test We found it to be rather less than pig gland but it is a substantial amount and yet in this alkaline extract there is virtually none at all It is to be assumed that enzyme action of some sort has occurred under these conditions which has resulted in destruction particularly of the material active in the Sayers test but although we have done a few preliminary experiments on this point we have no clear evidence on the nature of the enzymes

Pincus May I interrupt at this point and ask about the dehydro epiandrosterone? Is this determined chemically or purely on the basis of a color reaction?

Young It is on the basis of color reaction

Pincus I should like to point out that the color reaction may not be specific

Young Yes I think, as Dr Vogt (13) has pointed out one has to be extremely careful about basing any estimation with respect to eosinopenic activity on nonspecific proteins It seems that non specific protein substances are capable of reducing eosinophils and it appears improbable that the alkali heated intermedin does possess physiologically significant activity with respect to eosinophil reducing activity or any other type of action I do think it is of interest however, that Collip (5) in his early work on ACTH assayed by adrenal weight maintaining activity in hypophysectomized rats and observed that the preparations he had available were remarkably stable to heating in alkali even at pH 10 or 11 We have the intention which we have not yet been able to carry out of going over those earlier findings of Collip and assaying ACTH by adrenal weight increasing activity as he himself did

Our own interest in adrenal weight increasing activity of ACTH preparations began when we found that the activity of some relatively purified ACTH preparations that is purified on the basis of the Sayers test was relatively poor and that conversely the adrenal weight increasing activity of some other extracts that we were able to prepare which had minimal activity in the Sayers test was substantial (1) In our test for adrenal weight increasing activity we hypophysectomized rats and left them for a couple of weeks or so for the adrenal glands to atrophy and then administered material three times a day for three days interperitoneally the animals being killed on the fourth day and the adrenal glands being weighed We regarded that as a pretty rigorous test for adrenal weight increase activity since the atrophy occurring during the two weeks after hypophysectomy was quite substantial

Long Did you test the ascorbic acid depletion of rats two weeks or two or three days after hypophysectomy?

Young We did the conventional Sayers test In these experiments we found that a crude alkaline extract of ox anterior pituitary gland which we had used in other work on diabetogenic action among other things was remarkably ineffective in the Sayers test and we are still not clear why this is so This extract is prepared from fresh ox anterior pituitary tissue by grinding with saline at

find some differences between the AA and AW tests of the histological activity of what I might call conventional ACTH

Pincus I do not understand your ratios in Table V how do you obtain them?

Young An Armour preparation 84/85 U was taken as a standard in the AA and AW tests (actually it was 2 5 International units per mg) and we compared the other substances in terms of that standard That process is open to a great deal of criticism but if one is comparing activities in different types of response of this sort I do not think the ratios can mean anything quantitatively precise except that they were so large that they did indicate there was a qualitative difference between the types of extract

In a histological assessment of the adrenal glands we thought we might find some difference between the material that was active in the AA and AW tests However when we treated the hypophysectomized rat with an AW preparation (growth hormone in this case) the adrenal gland was found to be much more normal than when larger doses of conventional AA active ACTH were administered That has been a pretty consistent finding If we give a large dose of AA material we find a distribution of lipid reminiscent of that which is seen in the intact animal under conditions of stress and which we do not obtain with FGS the crude preparation growth hormone or other AW active materials

Pincus Is this done by intravenous administration?

Young No these tests are all carried out by intraperitoneal administration

Ralli Did you say that the adrenal weight anterior pituitary factor had some growth hormone in it?

Young Yes it did We have in fact used growth hormone as an AW preparation We also found there was a difference between the two types of active material with respect to stimulation of mitotic activity

When we examined the number of mitotic figures in an arbitrary area of adrenal gland of a normal hypophysectomized rat treated with a dose of adrenal ascorbic acid reducing material and also of one treated with growth hormone we found there was no significant change in the size of the cells (18) The FGS material or growth hormone would bring back the mitotic activity appearance of the adrenal cortex to something approaching normal which we were unable to do with any reasonable dose of ascorbic acid reducing active material (Figure 20) In fact if we gave any large dose of the ascorbic acid reducing material we always obtained abnormal

TABLE V

Relative Activities of Pituitary Preparations in Terms of the Activity of Preparation 84/85 U

Fraction	Total dose (mg)	Equivalent to standard in		Ratio of AW to AA activity
		AA test	AW test	
FGS A	90	0.0005	2.6	5200/1
FGS B	180	0.0012	2.9	2400/1
Growth hormone	22	0.0024	2.0	830/1
84/85 U	5	5.0	5.0	1/1
84/85 H	15	20.5	2.0	1/8

The crude alkaline extract that I have been referring to is called FGS in Table V In the first paper we published on this (1) we calculated a ratio of activity in the ascorbic acid reducing (AA) test as compared with activity in the adrenal weight increasing activity (AW) test that we had utilized These figures show that the adrenal weight increasing activity is significant without there being anything that can be regarded as comparable ascorbic acid reducing activity

This particular crude extract the FGS was quite rich in growth promoting activity In the early stages of our work we had used it for the preparation of growth hormone We therefore were led to test growth hormone for an action in the adrenal weight increasing test particularly since there had been suggestions by Dr Selye and others that it might have some action of a mineralo cortico tropic type We found that it was indeed active in adrenal weight increasing tests although most of the preparations of growth hormone that we had were rather more active in the Sayers test than the crude FGS extract had been We found in general weight for weight that the growth hormone we were utilizing was about five times as active in the Sayers test that is about 14 gm of the material was equivalent to one International unit of ACTH in the Sayers assay Whether that figure is significant or not I should not like to say I think it probably is not

We have attempted to investigate the type of action on the adrenal cortex of these various preparations the FGS and the growth hormone because in the early stages of this work we did

distribution of lipid which we did not find with the adrenal weight increasing preparations

Selye Did you obtain an actual increase above normal in lipids with the adrenal weight factor?

Young We have done very little in the way of chemical estimation of lipids

Long Have you determined the adrenal cholesterol and was it normal in the glands treated with the AW factor?

Young Again we have done very little on that but in a few estimations in the early stages we found an essentially normal cholesterol When the adrenal weight increasing material and the ascorbic acid reducing activity were given simultaneously there was certainly some evidence of summation of effects with respect to adrenal weight increasing activity but none with respect to mitotic stimulating activity that is the latter seemed to be more clearly associated with the factor that was not effective in the Sayers test

It is true that large adrenal glands may be produced by giving large doses of corticotropin but in our studies the histological picture of these glands was quite dissimilar to that seen after the administration of adrenal weight increasing preparations and in any case as I already emphasized the adrenal ascorbic acid reducing activity of our preparations was so small as to be virtually negligible

We have not gone very far with the fractionation of material that is active in the adrenal weight increase test since a good part of our energy has been expended in the direction of the ascorbic acid reducing factor and we have not progressed very satisfactorily with that either However in the experiments on the fractionation of ascorbic acid reducing activity with ion exchange resin particularly amberlite IRC 50 which Dr Stanford Moore (19) has used so successfully we found that fractions which ran fast on a column of amberlite IRC 50 produced very large adrenal glands in the AW test although they showed only slight AA activity (20)

When any crude preparation of ACTH is fractionated on a column of amberlite IRC 50 at pH 6 7 by the method which I need not describe in detail the adrenal weight increasing material is less retarded through the column than is ascorbic acid reducing activity That has been a reasonably consistent finding in fractionation on the amberlite IRC 50 (Figure 21)

We have not obtained complete separation of AA and AW activities by using methods of this sort and it is conceivable if two separable substances overlap in activities that we shall never do so

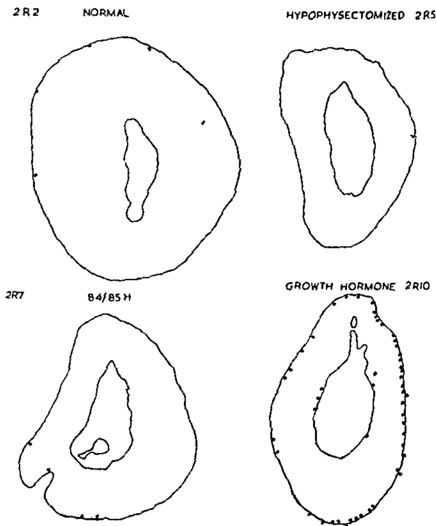


FIGURE 20 Mitotic figures counted in 12 sq mm of adrenal cortex The two lower figures give results for hypophysectomized rats treated with AA preparation 84/85 H (0 5 mg totally equivalent to 1 7 International units) or with growth hormone (20 mg totally) The hormones were given in divided doses thrice daily for three days intraperitoneally Reprinted by permission from Cater D B and Stack Dunne M P The histological changes in the adrenal of the hypophysectomised rat after treatment with pituitary preparations *J Path & Bact 66 119 (1953)*

However there were possible alternatives in interpretation that had to be considered It might be suspected that AA active fractions would be more effective in the AW test when mixed with protein or other material which would be present in fractions coming through the column early These materials might delay absorption through the peritoneum and might therefore enhance the activity of the material in the AW test or perhaps protect it from extra vascular inactivation because when prepared by different methods there appear to be very different susceptibilities of materials to extravascular inactivation

Thus in many of our experiments we have attempted to slow down to a very substantial extent the rate of absorption of these materials in the AW test by mixing them with various types of material We have used inert protein (serum albumin or casein) precipitated by tannic acid and in some cases the beeswax Arachis method introduced by Bruce and Parkes (21) In all cases we find that the difference between the peak of AA and AW activities in these experiments is still maintained that is one cannot by slowing the absorption alter the apparent peak of AW activity We are disinclined to believe on the basis of that evidence that these effects are merely due to differences in the rate of absorption of material Certainly we still find the differences for instance when the absolute value of activity may be increased by as much as twentyfold by mixing with various inert substances designed to delay absorption The fractionation procedures that we have carried out so far have been inadequate to differentiate between growth hormone and the AW active material

I should like to refer to some experiments of Rinfret (22) and Forsham (23) that have been published within the last year which may be relevant to our own findings They report that in extract of horse pituitary glands which is poor in ascorbic acid reducing activity (although they do not give precise figures) will neverthe less maintain the adrenal glands of the hypophysectomized rat The intravenous administration of this extract in man in contrast to that of conventional ACTH leads to almost no immediate increase in adrenal steroid secretion as estimated by urinary 17 hydroxycorti costeroid and 17 ketosteroid excretion and induces no change in circulating eosinophils

When a human subject was treated with this adrenal weight increasing preparation for five successive days the subsequent injec tion of AA preparations (conventional ACTH) led to an increase in the urinary excretion of 17 hydroxycorticosteroids and 17 keto

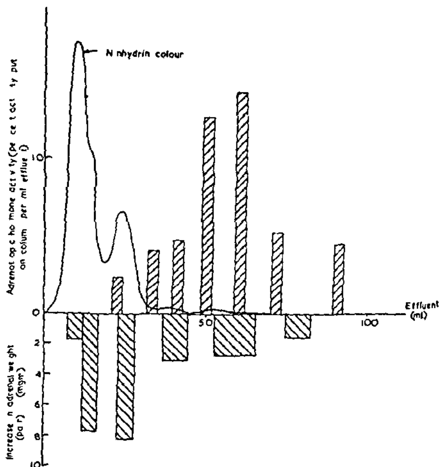


FIGURE 21 Chrom itogr im of crude corticotropin on amberlite IRC 50 (200-400 mesh) in 0 2M sodium phosphate buffer pH 6 69 containing 0 2 per cent phenol and 0 5 per cent thiodiglycol 475 mg of crude corticotropin was extracted with 9 5 ml of the buffer and 8 ml of the solution put on a 2 cm x 29 cm column Weight of untreated adrenals was 9 0 mg The erect hatched areas represent AA activity The dependent hatched areas represent AW activity Reprinted by permission from Dixon H B F Stack Dunne M P Young F G and Cater D B Influence of adrenotropic hormone fractions on adrenal repair and on adrenal ascorbic acid *Nature 168 1044 (1951)*

Young We have not studied that but we should have noticed it in some experiments in which they have been given simultaneously

Conn Have you compared mitotic activity in any other organ with that in the adrenal under the same circumstances?

Young We have not done any consistent survey in this connection but we have observed in other aspects of our work with growth hormone and with crude FGS that there is certainly mitotic activity in other glands Certainly one sees it in the islands of Langerhans of the pancreas and also in the pituitary gland in normal animals

Astwood Do you find an increase in thyroid and gonad weight under these same conditions?

Young We do find consistently an increase in gonad weight which has rather disturbed us because there is no evidence that our growth hormone is free from follicular stimulating hormone (FSH) We find an unimpaired effect on the adrenal weight in castrated rats and it appears as far as we can tell to eliminate the possibility that the adrenal weight increasing action is mediated by the secretion of androgens by the testes

Astwood You interpret this increase in gonad weight as being due to an effect of growth hormone or to a hitherto unrecognized gonadotropin?

Young We should have performed the experiment of testing for action on the gonads in the absence of the adrenals because of the possibility that androgen production by the adrenals may have been stimulated under these conditions However this has not been done as yet and until it has we shall have to accept the simple interpretation of our findings which is that the growth hormone is probably contaminated with FSH

Astwood Do you have evidence that the adrenal weight factor is indeed not growth hormone?

Young My own feeling is that it is not but we have no evidence that it is not or that it is

Long I assume that you have not fractionated growth hormone on amberlite columns to see whether you would obtain the type of separation that occurs with ACTH?

Young We have tried preliminary experiments of that sort but we have not been very successful so far in finding the conditions that would be appropriate

Long Dr Li what was the effect of the highly purified growth hormones on the adrenals in the long term experiments?

steroids which was two to three times those consistently observed in the same subjects before treatment with AW preparations Purified conventional ACTH and growth hormone in their hands both failed to induce an effect comparable with that of their AW preparation What the relation of their observation is to ours I am not certain although they may conceivably be related

Bush (24) has carried out some experiments with some of our preparations intravenously administered to the hypophysectomized rat and has found that the administration of the AA preparations increases the amount of corticosterone in the adrenal venous blood and that the administration of AW preparations under these conditions causes no increase He has observed that this is true whether the material is administered intravenously or whether it is given some days previously by subcutaneous or intraperitoneal routes These findings could conceivably be related to those of Rinfret and others although unfortunately we have not been able to follow them up

Pincus As I remember Bush found that he did not obtain the same effect when he followed the AW with the conventional AA preparation, and that there was no indication of an enhancement of corticoid secretion Is that so?

Young I believe he found no enhancement under those conditions

Ingle How much increase in adrenal weight did you find after the injection of the adrenal weight factor for four days?

Young There is a return to the normal weight of the adrenals Under some conditions however that is the exception rather than the rule The adrenals usually weigh up to 18 or 19 mg in the size of rats we have been using with the controls weighing about 10 to 12 mg

Li What happens to the thymus weight?

Young We have not done a great deal on thymus weight but there is no significant change in these AW tests I should have emphasized that we have used rather older animals and perhaps we could miss changes in thymus weight that might be more obvious in young animals

Long Is there any effect on eosinophils of those preparations?

Young We have not looked into that

Selye When you give the two hormones ACTH and growth hormone simultaneously do you obtain an inhibition of the ACTH involution by your AW factor?

Dr Li very kindly sent us a small sample of his purified growth hormone and this gave us the best peak fraction of any material we handled however it still contained a small amount of material which could be removed by the countercurrent distribution and it makes one extremely hesitant about assuming that growth hormone is pure The type of evidence that would be useful to us at any rate with regard to the presence or absence of adrenal weight increasing activity and growth promoting activity would be of the types we have been pursuing for some time in respect to diabeto genic activity If we could find that under all conditions of partial inactivation by various treatments that any disappearance of adrenal weight increasing activity was associated with a comparable disappearance of growth promoting activity we might feel that there was an association between the two if we could separate them completely we might feel there was no association However at the moment we are lacking in any evidence on this point and therefore I should prefer to await further evidence as to whether growth hormone is or is not a responsible agent

Loeb Dr Young was the material you used beef in origin?

Young Yes

Loeb Dr Li did you also use beef?

Li Yes Dr Young what system do you use for the countercur rent distribution?

Young *P* toluenesulfonic acid and 2 butanol

Pincus May I ask whether a similar administration of a highly purified ACTH gave you very different results? In other words in hypophysectomized animals do you observe the growth of the adrenal?

Li Oh yes it was very marked with almost any purified prepara tion although the injection vehicle makes a difference in result When the injection is administered in saline solution there is no gain in adrenal weight or only a very slight gain However when the preparation is suspended in beeswax peanut oil according to the method of Bruce Parkes and Perry (27) there is a very marked increase As a matter of fact in collaboration with Dr W R Lyons (28) we have obtained evidence of progestogen secretion in these greatly hypertrophied adrenals When the injection vehicles which result in delayed absorption are used an adrenal weight of about twenty or even forty times the size of the controls may be obtained

Young I would not disagree with that Substantial enlargement of the adrenal glands may be produced with what we may call

Li In 1951 we summarized our data accumulated over a period of 12 years on the relationship of growth hormone to adrenal weight changes in hypophysectomized rats (25) We found no evidence that the adrenal cortex is stimulated by growth hormone It is true that the size of the adrenal increased somewhat in some cases but this increase in adrenal weight seems to be almost solely due to the enlargement of the adrenal medulla In an experiment in which growth hormone in a total dose of 555 mg was injected for a period of more than a year to young hypophysectomized rats the body grew to about ten times the original size but the adrenal weight when calculated in proportion to body weight decreased 31 per cent as compared with the controls We have been following Dr Young's work with keen interest with respect to the possibility of some association between the AW factor and growth hormone and we have tried to fractionate growth hormone as far as we could So far we have been unable to find that there is any substance which can be fractionated from growth hormone that has any effect on the adrenal. Hence we feel very strongly that the growth hormone prepared in our laboratory does not contain an adrenal weight factor and any changes in adrenal weight produced by it must be due to contamination by an adrenal stimulating substance and not primarily to growth hormone

Young Have you observed mitotic activity in the adrenal glands?

Li We have seen no evidence of mitotic changes comparable to yours

Young I am sorry that the work on mitotic activity has not yet been published

In relation to diabetogenic activity of growth hormone so far we have been unable to differentiate it from growth promoting activity In regard to the action on the adrenals we have no evidence one way or the other to say that the two go together or that they differ All we know at the moment is that all the preparations of growth hormone which we have handled do have mitotic stimulating activity and adrenal weight increasing activity

I should be very apprehensive about saying that any preparations of growth hormone are pure During the last year Dr J G Pierce of the University of Cambridge (26) has developed a countercurrent distribution system for the purification of growth hormone and has managed to remove from all the preparations of growth hormone we have had some small amount of material which is not in the main growth promoting fraction This small fraction has not yet been tested for biological activity of various types

TABLE VI

Effect of Acid Hydrolysis on ACTH Activities by Two Methods of Assay

Preparation	Ascorbic Acid Depleting Activity		Adrenal Weight Activity*			
	Intravenous injection with saline	Subcutaneous injection with gelatin	Dose†	No of rats	Adrenal**	Thymus**
	USP units per mg	USP units per mg	Mg		Mg	Mg
Untreated	42	43	0 1	19	28	75
Acid hydrolysate	38	53	0 1	10	16	218
			0 0	25	11	228

*Injected into male rats hypophysectomized at 40 days of age once daily over a four day period beginning four days postoperatively
†Injection solvent 5 per cent beeswax in peanut oil
**Control adrenal 11 mg thymus 228

of adrenal weight from 11 to 16 mg produced by the hydrolyzed material but with the untreated material there is an increase which may reach 28 mg during the four day period

Young We have results which are quite in agreement with that We compared preparations before and after a partial acid hydrolysis and found as Dr Li has also that there was a diminution in the effect of these materials on the adrenal weight activity However I am disinclined to quote that as evidence in support of the general view that there might be more than one factor because in this case we are arguing on a quantitative difference and the question of rate of absorption might conceivably be a factor which would be more difficult to control there than in the other preparations that we have had in which there were extreme differences in activity

Dr Li have you ever tested a crude alkaline extract of beef pituitary for adrenal weight increasing activity?

Li Yes we have tested some but the adrenal weight increase is not impressive

Young What about the ascorbic acid reducing activity?

Li We do not have very extensive data concerning the ascorbic acid depleting activity of this particular extract but it is my impression that it was very low

ascorbic acid reducing factors Whether the histological picture there is comparable to that seen in animals treated with AW preparations is I think a matter for discussion The point I wish to emphasize is that we do obtain a very substantial effect on the adrenal weight with material which is virtually lacking in ascorbic acid reducing action I do not think that the question of rate of absorption or any other factor is likely to account for the findings If we could obtain material that is completely lacking in any action in the Sayers test that would be more satisfactory, but when we administer very large doses of any preparation of growth hormone we often get a very small effect in the Sayers test which I am inclined to think is not significant it is so small that it probably does not account for the substantial adrenal weight increasing effect of these preparations

Li In one of our experiments a female rat was hypophysectomized at 27 days of age Three days later injections of 30 μg of α corticotropin were begun which continued daily for nine days At the end of that time the adrenal weighed 22 mg and when compared with that of a hypophysectomized control animal it appeared hypertrophied and overstimulated The adrenal from the hypophysectomized control animal was injected with beeswax peanut oil and weighed 4 mg

When purified ACTH is hydrolyzed with 0 2 M hydrochloric acid in a 0 1 per cent solution and refluxed for three hours some hydrolysis takes place This preparation apparently retains its ascorbic acid depleting activity but the adrenal weight increasing activity is greatly reduced even when it is injected in a beeswax peanut oil suspension

Young Did I understand you to say the ascorbic acid reducing activity was the same after hydrolysis? I thought you said previously that it was increased

Li It was hydrolyzed much less In this case the hydrolysis was carried out with refluxing for three hours The same thing occurs whether the refluxing is for 40 minutes or for one hour

White Have you given this acid hydrolyzed preparation by continuous intravenous drip in your rats to see whether you can really get the adrenals to grow?

Li Not in the experiment I have been citing Table VI gives the assay data for the acid hydrolyzate which was obtained under the conditions just described We observed that the untreated material reduced the thymus weight markedly but that the hydrolyzed material had scarcely any effect There is a slight increase

hours before removal of the adrenal for ascorbic acid determinations were also used in our experiments

Astwood I do not think that the three hour subcutaneous method agrees quite as well with clinical effectiveness as has been suggested

Long I wonder whether there are any conventional ACTH preparations that are not rapidly inactivated in the blood stream following injection We are interested in this problem for a good many reasons not entirely related to their chemistry Recently one of my colleagues Mr A Brodish devised a method of cross circulating rats so that as much as 100 ml of blood was exchanged in an hour The purpose of this was to obtain some measure of the output of ACTH under various conditions

Pincus Do you mean of the normal pituitary?

Long Yes If a normal rat is put in cross circulation with a hypophysectomized animal the fall in the adrenal ascorbic acid of the latter is a measure of the amount of ACTH that has been liberated from the pituitary of the normal animal One complication is the question as to how much and how rapid is the inactivation of ACTH that is released since it would appear that this can occur with great rapidity Such rapid inactivation would obviously give too low values when judged by the depletion of adrenal ascorbic acid in the hypophysectomized animal We have attempted to assess this by establishing cross circulation between two hypophysectomized rats and giving ACTH to one The depletion of the adrenal ascorbic acid of both animals is then determined and it is easy to see that when small amounts of ACTH of the order of 5 µg are administered that considerable inactivation of ACTH occurs during the period of cross circulation Whether this is also true for the naturally secreted ACTH cannot be answered at present

Thorn The ACTH in those experiments was given intravenously?

Long Yes

Pincus Dr Li in the experiments on the thymus weight was the material given subcutaneously in gelatin?

Li No it was given in beeswax peanut oil suspension We found it more effective than the 16 per cent gelatin medium

Pincus The rate of movement of the material out of the beeswax and into the blood would be an unknown factor

Li The process would be very slow

Pincus Thus the degree of inactivation which you speak of would be purely a function of how much actually gets in?

Young Dr Li was the material in your crude alkaline sterile extract?

Pincus In Table VI, the data on adrenal weight and thymus weight, show that the untreated material increases adrenal weight and that it is quite thymolytic in its activity By these criteria the acid hydrolyzate is just an ineffective preparation The acid hydrolyzate is administered over a period of four days as compared with just a few hours for the ascorbic acid test Therefore, perhaps it is inactivated You have shown that ACTH preparations may be inactivated by a number of tissues and we have found such inactivation in blood (29) but what I am wondering is whether the hydrolysis simply does not prepare a substance for the inactivating enzymes?

Li Dr White pointed that out

Pincus The other question I should like to ask is do you have preparations which are differentially inactivated? You did some studies on inactivation by tissues Do you have one ACTH which is very rapidly inactivated by tissue and another which is very slowly inactivated?

Li There is some indication to that effect but it is not very clear cut

Dorfman How many animals were used in those ascorbic acid depleting activity tests?

Li For the ascorbic acid test there were at least 7 animals per group with two or three dose levels and sometimes as many as 33 animals in one level

Astwood In line with Dr Pincus suggestion Drs Forsham Renold and Frawley (30) in 1951 showed that some preparations suffered local inactivation and that others did not More recently Forsham and co workers (31) have shown that whereas corticotropin A type preparation which has not been subject to hydrolysis is not inactivated subcutaneously treatment with pepsin to yield what Merck calls corticotropin B type preparation makes the material very ineffectual subcutaneously suggesting that it is rapidly inactivated at the subcutaneous site whereas it maintains its activity in the Sayers test

Young Have you yourself any evidence on that point?

Astwood No we have not

Young Has anybody ever confirmed that view of Forsham s?

Astwood Corticotropin assays in rats using subcutaneous injections of gelatin and adrenal ascorbic acid depletion in three hours are in agreement with that view

Li Subcutaneous injections of 16 per cent gelatin injected three

weight protein At the time we did the experiment we did not have the ascorbic acid depletion test I remember that Dr White went to great pains to calculate it in terms of body weight at a dose level of 20 μg per day we were able to obtain a fair adrenal weight increase I think Dr White and Dr Long will remember that We are not able to obtain an adrenal weight increase with our most highly purified ACTH preparation which assays 200 I U per mg according to the ascorbic acid depletion assay without using de layed absorption

White This is the partially hydrolyzed product?

Li It is unhydrolyzed but it has been submitted to the oxycel lulose adsorption procedure

Long Dr Astwood summed that situation up very well a year or two ago at this conference He said that all we isolated was ballast protein with the ascorbic acid depleting factor attached or words to that effect

Young Dr Li if you mix the purified ACTH with what Dr Long has called "ballast protein" do you in fact increase its AW activity?

Li No if you mix it with growth hormone FSH or pituitary extract you do not obtain the adrenal weight increase

Young In general we find that the adrenal weight increasing effectiveness of our preparations is obtained by mixing them with an inert protein particularly if we make the materials relatively insoluble

Li It is a very soluble protein it does not seem to me to be a matter of solubility

Young I think it is perfectly reasonable to suppose that the adrenal weight increasing activity of these materials will be in creased if there is slower absorption over a period of time and that in fact is what we find It is conceivable that the adrenal weight increasing activity of the protein hormone may have been due to the fact that there is 1 per cent ascorbic acid reducing material and 99 per cent protein Dr Li you do not find that your more recent experiments agree with that?

Li No

Long I should like to admit to heresy here for the sake of argument It seems to me that one of the main points of this discus sion rests on the comparison of ascorbic acid depletion assigned to a certain activity of the gland against adrenal weight Are we so sure that the ascorbic acid depletion method is the infallible test under all circumstances? Actually how much do we know about

Li No We used an alkaline solution at around pH 11 which might be different from an extract made at pH 5

Young There may be something peculiar about this crude alkaline extract which we prepared We used it as a standard for a good many years in the early work on diabetogenic activity and found that it was essential to maintain approximately sterile conditions from the early stages so that we could obtain diabetogenic activity We Seitz filtered the material for storage purposes if we did not do that we lost diabetogenic activity It is that material which we used subsequently for our AW test

There is a recently published paper by Winter Brink and Folkers (32) in which they failed to find good adrenal weight increasing activity in a crude alkaline extract They say that their material had less than one unit of ascorbic acid reducing activity per milligram I had the opportunity of discussing this point with Dr Winter the other day but he was not very clear as to just what the activity had been I do not think they assayed it very closely for AA activity and they had had trouble apparently with toxicity of this material which we certainly do not have with our crude extract Thus I wonder whether the whole question of sterility and bacterial action may not be of very much greater significance with these crude extracts than we have thought in the past Dr Astwood you have not used crude extracts of this type have you?

Astwood We have not studied the AW phenomenon at all

White It might be worth while to ask Drs Young and Li whether either of them has succeeded in further fractionating an alkaline extract of beef pituitary tissue and obtaining a so called ACTH like protein The reason I ask the question is that it was our impression many years ago that in the case of the beef pituitary one starts first with a gland that does not have a great deal relatively speaking of ACTH and secondly that alkaline as compared to acid extraction is relatively inefficient for getting out what ACTH is present I wonder whether one can really isolate from such a crude alkaline extract something that resembles a 20 000 molecular weight protein of ACTH or anything else in the way of a purified preparation

Young We have not tried that As I say the material is so lacking in ascorbic acid reducing activity that we have not considered it but it might be well to look into it as a source of material which is AW active

Li Dr White brought out a very interesting point We agree that the adrenal weight activity is good in our 20 000 molecular

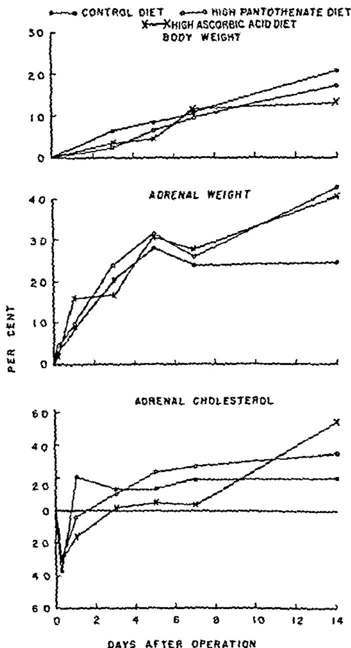


FIGURE 22 Average percentage change in body weight adrenal weight and total adrenal cholesterol in male rats from time of operation (unilateral adrenalectomy) to time of sacrifice as influenced by a normal diet and diets containing large amounts of pantothenic or ascorbic acids

the alterations in ascorbic acid and adrenal glands other than in the rat? Certainly, in the guinea pig we have considerable difficulty in establishing the relationship of ascorbic acid to the activity, particularly if the animal happens to be deprived of vitamin C. Nevertheless, under those circumstances we do know that the adrenal is active.

Young I entirely agree with you, Dr Long. If the Sayers test is assumed to indicate something, whatever it is, in the rat we are interested to know whether, under those conditions, it is also responsible for the adrenal weight increasing effect.

Long I should like to extend that further. I think it has been purely a matter of chemical convenience that ascorbic acid was selected as the method of assay. One can present a much better argument for following the changes in the adrenal cholesterol as being much more closely related to the actual formation and release of the hormone, and yet there is practically no work being done on the association of adrenal weight changes with changes in the adrenal cholesterol. In fact, I can point to many examples where the ascorbic acid change can be disassociated from the adrenal cholesterol. I shall refer again to the situation in the guinea pig and there are other circumstances too. Recently we have been simultaneously determining the eosinophil count, adrenal cholesterol and adrenal ascorbic acid. Without any difficulty whatever I can present to this group an idea, supported by experimental data, that there is a factor in the pituitary that lowers adrenal cholesterol and does not lower adrenal ascorbic acid, or any other combination you may wish to consider. However, the whole emphasis here seems to be placed on the ascorbic acid as the infallible indicator. It may be extremely useful as an assay method, and I grant the convenience in doing it in the rat, but do we know that the adrenal ascorbic acid in other species does change in response to ACTH? We have done a few experiments in which we have performed a unilateral adrenalectomy on cats and on rabbits, spacing the removal of the two glands two or three hours or more apart. We have not done a great many of them, but certainly, in contrast to the rat, changes in ascorbic acid after unilateral adrenalectomy in larger animals are small within an hour or two compared to the 50 per cent drop obtained in the rat. It is hard to believe that the adrenal pituitary system has not been activated to a very high degree when a laparotomy is done in these other animals.

Ralli May I present some work we have done along this same line in the normal rat rather than in the hypophysectomized rat?

studies with adrenal slices that any of these various preparations actually produces a different kind of steroid?

Dorfman We have started to study that point We have used various ACTH preparations a highly purified preparation from Dr Astwood and two crude preparations in an *in vitro* adrenal slice system Our experience indicates that there is a correlation between ascorbic acid depleting activity and the quantity of hydrocortisone produced

Young We have not done a great deal on the question of adrenal cholesterol but the possible working hypothesis we have had what we have called the AW factor may be concerned with the deposition of cholesterol in the gland and not with production of steroids from it

Ingle There are grounds for questioning the reliability of these tests done from one laboratory to another even when the same standards and the same general procedures are employed During the past two years I have seen many data on assays from different laboratories Preparations which I have received from Dr Li from Dr Astwood from Armour and from Merck have all been re assayed in the Department of Endocrinology at the Upjohn Company and it was rather rare to find agreement between them Moreover there were two separate groups at Upjohn s doing these assays and each group learned from the other They were using the same strain of rats and were duplicating the procedures as nearly as possible During this past year they carried out a study with Dr George Sayers taking several preparations of ACTH and assaying them as unknowns For a few preparations there was a certain agreement but there was significant disagreement among others The adrenal weight method has never been a very reliable method from the standpoint of sensitivity In respect to technique alone there are bases for considerable disagreement

Pincus We have been trying to develop a perfusion method as an assay method and Mr Macchi in our laboratory at the Worcester Foundation has worked on this for several years As Dr Dorfman has pointed out while there is a correlation between the ascorbic acid depleting activity and the amount of steroid produced we also find that from one day to the next we do not always obtain the same result On one day the ACTH will give us so many milligrams of steroid per hour and on the next day in similar glands it will give us one fifth or one tenth or three times as much Was there not an attempt by Haynes (34) to try to develop an *in vitro* assay and did he not also find this great variation?

The left adrenal of the rat was removed, weighed and the cholesterol content determined At varying intervals of time after unilateral adrenalectomy the right adrenal was removed and the same determinations made These data are preliminary and are part of a study on the effect of nutritional fractions and stress on adrenal cholesterol Each point in Figure 22 represents the average of groups of nine or ten rats The upper section of the figure shows the body weight the middle section the adrenal weight and the lower section the adrenal cholesterol of rats at intervals varying from six hours to 14 days after unilateral adrenalectomy Data are presented from animals on (*a*) a control diet adequate in all the nutritional fractions required by the rat and shown by solid circles (*b*) this diet plus 4 mg of pantothenate per 10 gm of diet shown by clear circles and (*c*) the control diet plus 100 mg of ascorbic acid per 10 gm of diet shown by crosses Following unilateral adrenalectomy there was a sharp drop in the adrenal cholesterol in the rats in all three diet groups The restoration of cholesterol at 24 hours was greatest in the group on the control diet but from the third day on the group on high pantothenate showed a greater increase in adrenal cholesterol content than the controls The restoration of adrenal cholesterol in the group receiving ascorbic acid was retarded up to the seventh day but by 14 days in the animals observed up to the present the adrenal cholesterol content was greater than in the other two groups Interpretation of these data must be postponed until additional animals have been studied This type of experiment is we think a good index of the response of the adrenal to stress in animals conditioned in various ways We have published a report on the effects of diets deficient in pantothenic acid on the restoration of adrenal cholesterol following stress (33) In the pantothenate deficient rat both the initial cholesterol concentration and its resynthesis following stress were markedly depressed We have not yet studied the hypophysectomized animal but hope to do so later

We also observed animals deficient in pantothenate in which the resynthesis of cholesterol was depressed during the entire period of time following the stress The absence of pantothenate seems definitely to retard or depress the resynthesis of cholesterol in the normal animal

White With the new methods for identification of steroids are we not in a position to decide definitely which ACTH does what in terms of producing steroids? In that connection I wonder whether there is any indication through perfusion or incubation

looked for other criteria which we think might be more satisfactory such as mitotic stimulating activity and an adrenal histological factor I think some of the discrepancies that do occur are of a relatively small nature a few hundred per cent and might well be ascribed to differences of this type in the adrenal weight effect

Astwood I wonder whether Dr Young would not agree that the main difficulty here is a matter of terminology The adrenal weight factor is really one that restores the adrenal weight to normal in the hypophysectomized rat which is a small weight increase whereas corticotropin is a substance that usually produces a very large increase in adrenal weight This causes a difficulty in the nomenclature

The second point is that many things can change adrenal weight Probably all cells in the body benefit from thyroid hormone it has been shown that cortisone plus androgen will restore the adrenal weight to normal after hypophysectomy just as estrogen will act directly on the ovary Yet we do not speak of estrogen as a gonadotropin or androgen as a corticotropin Perhaps Dr Young will agree to regard the adrenal weight factor as not a specific corticotropin If he would so agree then I think the difficulty would be resolved

Young I think the word specific is interesting there I was rather careful to say earlier that I would treat the term ACTH as covering all pituitary substances affecting the adrenal gland and I would not suggest that the substance that we are concerned with was limited in the effects on the gland

Astwood You would not regard androgen then as ACTH?

Young I referred earlier to an experiment of Winter and Folkers (32) where methylandrostenediol plus cortisone did affect adrenal weight I would have to include these substances if I defined ACTH as an ACTH like substance I should limit my definition to pituitary substances

Long Have any of the people working with this factor produced an adrenal larger than normal for example in a normal animal? Can an adrenal two or three times the normal size be produced as can be done with the conventional ACTH?

Young In our experience that is very difficult to do I cannot quote an actual experiment at the moment but in the adrenal gland of the normal animal — not the hypophysectomized rat of course — a substantial hypertrophy can be obtained

Loeb I should like to say that in the salt depleted rat we obtain hypertrophy of the glomerular zone Whether the actual weight of

Dorfman There is a variation With the adrenal slices we still have difficulties once out of every three trials which is a very serious situation However if two concentrations of a standard and two of an unknown were run simultaneously I think we could assay ACTH for its ability to produce corticosteroids with perhaps as good a precision as when the ascorbic acid test is used

Thorn We never were able to develop a completely satisfactory assay for adrenal extract The only thing which saved the situation was the isolation and synthesis of crystalline steroid material

Dorfman I should disagree with that point

Ingle May I make a few comments about this general problem? We have tried to eliminate some of the obvious difficulties by giving hormones in continuous intravenous injections and yet we do not find the answers to such questions as has there been inactivation of the hormone after it has been tested by one method and then put into solution to test by another method? The Merck group have told me that if corticotropin B is diluted beyond a certain point there is inactivation Even though we deliver the hormone to the blood stream without inactivation we know nothing about differences in rates of inactivation by the body fluids or other factors affecting the efficiency of its action If in trying to decide whether we have a true discrepancy in ratios of activity of different preparations in various assays we could apply these two criteria simultaneously under the identical conditions it would be more simple to answer these questions However as far as the adrenal ascorbic acid and adrenal weight tests are concerned one is an acute test and the other requires much longer administration of the hormone We have used metabolic responses as indices for stimulation of adrenal secretion This may be more meaningful than either morphologic changes in the gland or ascorbic acid depletion but if we could measure the output of the adrenal steroids from the gland itself this would provide the best possible answer to the question as to whether there is more than one ACTH

Young I should like to make a comment which arises out of a discussion I just now had with Dr Loeb I should have perhaps emphasized that an adrenal weight assay is bound to be a somewhat unsatisfactory method because the weight of a gland can be altered by factors such as engorgement of the blood vessels Dr Loeb pointed out edema and there are other factors such as the accumulation of cholesterol and so on which will necessarily cause an adrenal weight test to be less specific than say an ascorbic acid reducing test We have been aware of this and that is why we

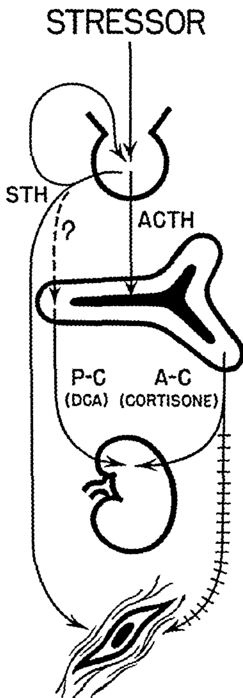


FIGURE 23

the gland increases I cannot recall Do you remember whether under those circumstances it is merely a redistribution of cellular activity or cellular hyperplasia in glands?

Selye I cannot recall actual weight The increase is not very marked although it can have a definite effect on the glomeruli

Loeb I do not think they are very much larger either but I am not sure

Li Androgen has corticotropic activity in the hypophysectomized animal only when injected on the day of operation If it is injected into a hypophysectomized rat at seven days postoperatively we obtain neither repair nor increase of adrenal weight

With regard to the point about an index of adrenal activity I think we should not forget that thymus reduction is a very good index of adrenal activity because it measures the functional activity of the adrenal

Pincus Has the Speirs method been sufficiently developed for ACTH assays?

Li Dr Pincus that *is a very touchy question For two or three* years Drs William O Reinhardt and Gerald F Hungerford have been testing our ACTH preparations for eosinopenic effect in both rats and mice Up until a year ago we obtained consistently good eosinopenic effect with some of our ACTH fractions but last summer when we purified ACTH further the eosinopenic effect in the hypophysectomized animal completely disappeared Now no matter how large a dose we inject we do not obtain this When cortisone is injected however an eosinopenic effect is produced When I mentioned it to Dr R S Speirs he thought the explanation might be that epinephrine is important for releasing the steroid through adrenal stimulation and he is at present testing one of our ACTH fractions

Thorn How long did you you give the ACTH continuously in the hypophysectomized animal?

Li The circulating eosinophil counts were taken either three or six hours after the ACTH injection

Thorn How long had the animals been hypophysectomized?

Li The injections in the hypophysectomized rat were given one day postoperatively *That has been our standard procedure for a* number of years and we always obtain good results *But this time* nothing happened The point is that when the ACTH is purified to this extent we no longer obtain any eosinopenic effect in hypophysectomized rats

pletely eliminated by adrenalectomy Indeed this effect cannot even be restored in the adrenalectomized animal by treatment with either cortisone or DCA Of course if such large amounts of DCA are given that it in itself causes nephrosclerosis then there will be renal damage but simultaneous treatment with STH fails to aggravate this further

The effect of STH upon the kidney may be related to an adrenal stimulating factor of some kind (The interrupted arrow is labeled by a question mark in the figure) In any event it is only this action of STH which could be considered to be adrenal mediated The pressor effect of large doses of STH which is manifest particularly in animals sensitized by unilateral nephrectomy and a high NaCl intake is likewise dependent upon the integrity of the adrenal cortex and quite possibly upon that of the kidney also

In discussions of this kind we have to keep in mind that the mineralo corticoid like actions which we obtained with impure pituitary extracts and subsequently with partially purified STH preparations (*including the most purified ones furnished by Dr Li*) simulate the mineralo-corticoid effects of DCA only in the sense outlined above These effects are of two entirely different kinds (*a*) the action upon connective tissue in general which is demonstrable even in the absence of adrenocortical tissue and (*b*) the effect upon the kidney which is dependent upon the presence of adrenocortical tissue I do not see how any single corticotropic factor could explain this complex picture

EDITOR'S NOTE Dr Selye would like to add the following comment to his remarks at the conference

In recent experiments with the granuloma pouch it was possible to demonstrate the production during systemic stress of some antiphlogistic factor which is of adrenal origin but which conditions the effect of cortisone as regards inhibition of inflammation In adrenalectomized rats bearing a standard granuloma pouch systemic stress (e g that produced by forced immobilization or a topical irritation arthritis of great intensity) has no effect upon the formation of exudate or granuloma Conversely under *similar conditions* in intact rats the resulting alarm reaction simultaneously with adrenocortical enlargement inhibits inflammatory phenomena in the pouch If adrenalectomized animals are maintained with DCA systemic stress still remains ineffective however if they are maintained with small doses of cortisone or hydrocortisone which in themselves cause no inhibition of inflammation systemic stress completely prevents exudation and diminishes granuloma formation just as in intact animals Obviously the antiphlogistic effect of corticoids is greatly enhanced by some extra adrenal manifestations of systemic stress This conditioning for antiphlogistic activity is reminiscent of

Selye Because we have discussed the adrenal weight factor which may have something to do with mineralo corticoid effects it might be instructive to review the manner in which our experimental arrangement differed from those which have been mentioned in this discussion

Figure 23 is a simple schematic drawing which summarizes what we have seen in our experiments Let me point out at once however that what I say about STH refers only to the purest preparation now available and not necessarily to the pure hormone itself We used Dr Li s highly purified growth hormone The latter may still be impure and the various findings may not have reflected the effects of a single compound but until we have positive evidence of this I shall continue to use the designation STH for the growth hormone preparations now available

The connective tissue cell at the bottom of the figure may be situated in subcutaneous tissue or in a joint or in any other location The effect of STH is exerted directly upon it Thus the stimulation of connective tissue and the augmentation of its reactivity to local irritants is a direct effect of STH *which does not have to be mediated through the adrenal cortex* It is similar to the effect produced by DCA and in this sense I consider it a mineralo corticoid like effect although it is not inhibited by adrenalectomy and hence cannot be interpreted as a result of adrenal stimulation

This effect of STH is also related to mineralo corticoids in that if subthreshold doses of DCA are given their effect is further augmented by STH due to some synergism between the two hormones On the contrary if the adrenal cortex is implicated in any way it is in an inhibitory capacity Animals treated with large doses of STH tend to discharge some ACTH from their own pituitary and the resulting gluco corticoid production has an inhibitory effect upon the connective tissue

Therefore in those experiments in which we gave STH to adrenalectomized rats the connective tissue proliferation and the augmentation of the inflammatory potential were even greater than in intact animals because the inhibitory effect of an antiphlogistic corticoid discharge was eliminated The stimulating effect on ACTH secretion of STH is indicated in the drawing by the arrow which reverts to the pituitary The other effect of this hormone is exerted upon the kidney Here it produces nephrosclerosis similar to that obtainable with DCA treatment It is this last mentioned action of STH which is completely abolished by adrenalectomy

The possibility of producing nephrosclerosis with STH is com

Bauer On the other hand it brings up the question of the sterility of *your STH preparations Without bacteriologic studies of these* joints one cannot rule out infection In the case of the rats in your experiments there are at least two possible sources of infection (*a*) the material injected and (*b*) the presence of *Streptobacillus moniliformis* in the rat colony In the case of the latter there may *result an infectious arthritis due to the Streptobacillus moniliformis* or its L form In animals insulted as much as these were it is conceivable there might result a blood stream infection with either the *Streptobacillus moniliformis* or its L form The base would be greatly strengthened if bacteriological studies ruled out the possibility of *Streptobacillus moniliformis infections* and showed that the STH was sterile

Selye If there is anyone who believes that in an STH treated animal the production of arthritis is due to the development of an infection at the site of formalin administration rather than to the *formalin itself he should certainly perform such sterility tests Per*sonally I am quite satisfied that formalin itself suffices to produce inflammation without invoking any such accidental infection

However be this as it may the STH preparation which we used did cause an increase in the ability of the connective tissue to respond with inflammation upon *local irritation* This was so not only in the topical irritation arthritis test but also in the granuloma pouch test The enhancing effect upon inflammation whether it is caused by formalin croton oil or even incidental infection does not depend upon the presence of the adrenals In the adrenalectomized animal the inflammatory potential can still be increased by giving STH or decreased by giving cortisone and that is the point I wish to emphasize Furthermore there is a proportionality between the dose of cortisone necessary for inhibition and the amount of STH required to prevent the inhibition All this is completely independent of the adrenal and has been demonstrated in bilaterally adrenalectomized rats

If there is some factor of infection in all this its existence would first have to be proved In any case the balance or antagonism between the prophlogistic STH and the antiphlogistic cortisone is demonstrable even after ablation of the adrenal while the effect of STH upon the kidney is dependent upon the presence of the suprarenal glands

Bauer I do not think there is any doubt that an increase in ground substance in the tissues will be obtained

that previously demonstrated in our laboratory for the blood sugar increase or the thymolytic action of these steroids All of these effects of glucocorticoids are augmented in adrenalectomized animals by systemic stressors

Bauer Do you obtain a connective tissue effect with Dr Li's preparation?

Selye Yes In fact Dr Li (35) published a paper in *Science* on essentially the same topic He also found that in adrenalectomized animals the extract does have an effect upon the joints and causes a type of chronic arthritis So this action could certainly not be mediated through the adrenal, and could not be explained on the basis of any among the corticotropic factors which we have discussed here

Long What is the effect on the connective tissue Dr Selye?

Selye First it causes a proliferation of connective tissue cells and fibers especially in the joint regions and secondly it augments the inflammatory response caused by local irritation of joints with some chemical agents for example formalin

Bauer I think you and Dr Li are speaking of two different things You are talking about an inflammatory process of synovial tissues while he is thinking of a degenerative lesion which takes place in cartilage which is more akin to degenerative joint disease or what is sometimes called osteoarthritis

Selye But both seem to be dependent on STH

Bauer The latter is a growth hormone effect We have no good evidence that it affects the formation of the matrix Would you agree with that Dr Li?

Li Yes I think it is probably the osteoarthritic type but we have no way of knowing at the moment

Bauer Did you say the most marked effect is in the case of cartilage particularly in the young growing animal whereas the situation is quite different in the adult animal?

Li It is quite true that growth hormone has been demonstrated to increase the collagen content of the connective tissue

Bauer If it is growth hormone effect the acromegalic should have rheumatoid arthritis yet we have known of no such case

Selye I have not studied arthritis in acromegalics Under the conditions of the complex derangement prevailing in acromegaly apparently STH produces the so called acromegalic type of arthritis rather than the rheumatoid type I was merely describing our experiments on rats

procedures If the same animal is now treated with STH the pressure can go up to 150 or even 200 mm of Hg and this is accompanied by kidney lesions

Li What are the sensitizing procedures used in connection with the high salt diet?

Selye One per cent sodium chloride solution instead of drinking water and a unilateral nephrectomy

Li You do not give a normal animal a normal diet? Do you get any hypertension?

Selye Not with the doses of growth hormone that we have given Also coming back to the importance of sensitizing procedures an increased blood pressure with nephrosclerosis periarteritis nodosa and death may be obtained by giving an overdose of DCA even in the absence of such sensitization I should like to call attention to the results of Dr Green (36) who has shown that approximately 200 µg of desoxycorticosterone per day suffices in a normal non sensitized animal to produce nephrosclerosis and hypertension In fact this treatment does not even have to continue until death because even a temporary DCA overdosage will cause so called meta corticoid self perpetuating hypertension and nephrosclerosis

Two hundred µg of desoxycorticosterone would correspond in a sodium potassium test to approximately 2 µg of electrocortin I think it is a matter of opinion whether this should be considered a very unreasonable high dose that could not be endogenously produced under any conditions

Long We have given many adrenalectomized rats DCA pellets and I do not think it would apply in that case

Loeb In what period of time?

Selye In about two months of treatment The pellet was gradually absorbed a fact which could be checked by inspection of the implantation site (36 37 38)

Long I should not speak for Dr Thorn but I believe that adrenalectomized humans have been maintained with DCA pellets in amounts sufficient to maintain body weight for a long time and without any evidence of nephrosclerosis

Bauer How would you account for the results Dr Li and his co workers have reported of experiments where growth hormone was administered for as long as 400 days with no higher incidence in nephrosclerosis than would be found in the control rats?

Selye These animals of Doctor Li s were not sensitized in any way and he administered much smaller quantities of STH than we did

Selye Maybe that is what sensitizes for inflammation in these regions

Bauer Such increased activity does not result in an inflammatory reaction Yet the photomicrographs of the joints you published showed practically nothing but polys in the exudate a finding that should always lead one to suspect a specific infectious process

Long Dr Selye did you say STH releases ACTH?

Selye Yes That is to say we obtain a rather pronounced adrenal enlargement with Dr Li s STH preparation in intact animals but practically none after hypophysectomy We assume that this corticotrophic effect of STH is dependent upon the release of endogenous ACTH

Long Did you obtain an enlargement of the cortex?

Selye Yes If the animal is highly overdosed with STH this may be a nonspecific stress effect but the point is difficult to prove

Long You also said it had a pressor effect I believe

Selye Yes

Long So animals treated with growth hormone do have an increase in blood pressure

Selye Yes if they are suitably sensitized by unilateral nephrectomy and a high salt diet

Incidentally the sensitization as such is another point worthy of comment Dr Bauer has mentioned in some of his writings that sensitization by high salt diet unilateral nephrectomy or castration are drastic procedures not likely to have any equivalent in clinical medicine Hence he expressed his doubts concerning the clinical implications that could be drawn from such animal work

Let me point out therefore that all these sensitizing procedures are necessary only if we wish to obtain nephrosclerosis and hypertension with the minimum amount of hormone during the shortest possible time Sensitization is merely a convenient experimental procedure However lesions such as nephrosclerosis and hypertension can be obtained with hormones even without sensitization

Long What increase in pressure do you obtain over and above what would be produced by other procedures?

Selye The other procedures do not cause any

Long But the blood pressure of the hypophysectomized rat is somewhat depressed to start with

Li Much depressed

Selye This was not done in hypophysectomized rats but the pressure of a normal animal amounts to about 130 mm of Hg when determined with our technique despite all the sensitizing

Long In 1945 Reiss and his colleagues (39) reported that the gonadotropic hormones increased cholesterol in the gland whereas ACTH decreased it I believe he obtained some increase in the adrenal size

Li With pregnant mare serum also

Young I think we differentiated from those particular preparations

Bloch King (40) has reported some marked increase of cholesterol synthesis in vitamin C deficiency

Long We observed that too in the guinea pig In 1940 Dr Collip reported (41) the pituitary effect on the adrenal medulla particularly the proliferation of dark cells It is a very interesting fact that if this is an adrenal medulla which is immune from anterior lobe influences it is almost doubled in size that is an over all 60 per cent increase in weight by growth hormone Has any other portion of the chromatin tissue been influenced for example the sympathetic ganglion?

Li We did not examine other areas

Pincus Is that not just about proportional to the increase in body weight or is it greater?

Li The medulla was enlarged disproportionately it was so huge that the cortex was practically pushed out

Young We found an effect on the medulla but nothing comparable with the adrenal weight we have observed

Selye I did not find any medullary changes Of course I did not treat my animals very long and there may be differences in the rats we used In any event I have not seen any adrenal medullary enlargement above that corresponding to the growth increment of the animal as a whole

Li This was a long term high dose experiment carried out for a period of up to 400 days in three series — two hypophysectomized and one normal

Long I recall that the effect on the brain and the spinal cord was very slight The growth hormone apparently does not make the brain twice the size If I remember your figures correctly the effect on the nervous system is very small

Li Yes

Long This effect on the medulla is an extremely interesting one since it is apparently not related to any stimulation of the central nervous system

Astwood Some years ago we did that experiment in a different way We had a sample of horse pituitary powder which would in a small dose *restore* the adrenal weight in hypophysectomized rats, but a dose 50 times larger did not increase the weight further

Young Rinfret (22) used horse powder, however we did not

Astwood I am sure the phenomenon you have described is absolutely reproducible

Young It is the significance of it that is the question You would regard it as a nonspecific effect of pituitary material?

Astwood Yes I would not call it a corticotropin

Young I hope for a revised definition of ACTH when I obtain pituitary substances which will affect the adrenal It would be included within that general category but it is a question of how specific this physiological effect is for the adrenal or whether it has a significant action

Astwood I am sure that any phenomenon associated with mitosis is physiologically important

Thorn In measuring the summation of the androgen effect and the weight increasing effect could you increase the total weight above normal with the use of the two preparations in combination?

Young We have not yet performed that experiment or really tried to go above normal in the hypophysectomized animal with our AW preparations My attitude is that of a biochemist who is interested in anything that could have physiological action and an attempt to isolate no matter what that substance is, seems to me the first step in trying to understand what function it may have in the animal economy I have no idea what the AW factor does As I say our working hypothesis has been that it might conceivably be a substance concerned with the deposition of cholesterol in the adrenal gland and it has an effect which seems not to correlate with the ascorbic acid reducing effect At the moment I hesitate to interpret it in terms of physiological action but I think it would be of interest to know what the substance is

Ralli When you did these studies on the mitotic effect of this substance did you examine any other tissues such as the liver to see whether it also was responding?

Young We did not examine the liver in those actual experiments but in some others we looked at the islands of Langerhans which particularly interested us In a number of other glands we found some effect but nothing very striking

Pincus One of the best ways of increasing adrenal cholesterol is to administer glucose to a rat

Long The question is is the increase in the size of the medulla greater than the increase in body size?

Li Yes only in very high dose normal mice In two instances we found that the medulla was increased in size in short term experiments but not in the hypophysectomized animals

Ralli Has there ever been any growth promoting assay of the activity of the type of pituitary tumor that is observed in acromegaly in the human?

Li There was one case some years ago Dr L W Kinsell sent us some blood from an acromegalic patient and we detected growth hormone in the blood

Ralli I have a case of acromegaly whom we have observed since 1931 He came in originally with xanthomatosis and a tremendously high cholesterol He is also a diabetic but it is difficult to know just how severe his diabetes is because he insists on taking 100 units of insulin a day regardless of what anyone says to him

Young Dr Li how do you estimate your growth hormone?

Li We use the tibia test for growth promoting activity and about 5 µg may be observed of growth hormone One can detect growth hormone even in the blood plasma (0 1 µg per ml) of the normal adult subject but in the acromegalic patient the content is much higher (20 µg per ml) according to this biological test

Young I do not think this is necessarily relevant to the present discussion but perhaps I might mention that Dr P J Randle in the Department of Biochemistry at the University of Cambridge has recently been estimating insulin in the plasma of various types of patients including acromegalics and has observed a diabetic acromegalic woman who as estimated by the rat diaphragm method seems to have about 15 times the normal amount of insulin in her blood I do not know whether anybody else has made an observation comparable to that The problem is that the plasma from this patient did not produce a fall in blood sugar in the diabetic hypophysectomized rat Our method was based on that of Groen (43) One complication is if growth hormone is added to normal plasma and tested by this method an increased insulin like action in the plasma is obtained

Li *In vitro*?

Young Yes

Dorfman What is the relative sensitivity between the tests *in vitro* and on the alloxan diabetic rat?

Young The alloxan diabetic hypophysectomized rat is about two or three times more sensitive but the amount of insulin activity – I

Li In experiments with mice carried out in collaboration with Dr William R Lyons of the Department of Anatomy University of California a high dose of growth hormone caused a body weight increase after 40 days of from 20 to about 60-odd gm and the medullas were enlarged as well

Astwood Dr Collip s factor affecting the adrenal medulla was effective by mouth

White In view of Dr Selye s statement that his hypertensive effect is adrenal mediated he has examined the same situation in adrenal demedullated animals to determine whether there is a non adrenal cortical effect in relation to some of these activities Is this medullary action real and has anyone done epinephrine assays of these hypertrophied adrenals?

Selye I have not performed any such experiments on adrenal demedullated animals Let me call attention to the fact however that my experiments do not even prove that the effect is necessarily adrenal mediated They tell us only that the action of STH upon the kidney does not occur in the absence of the adrenals It is possible that some adrenal factor may be present although the STH does not actually increase the secretion of this adrenal factor

Long Do you think we could produce an experimental pheochromocytoma by prolonged treatment with growth hormone?

Selye Are pheochromocytomas really common in acromegaly?

Bauer I do not know but I am sure of one thing the acromegalic may have adenomas in other endocrine glands

Loeb There is a syndrome of adenomatosis of the endocrine gland which may involve the pituitary parathyroid and islands of Langerhans but I think probably that is not the same as ordinary acromegly because changes in the parathyroid and islands of Langerhans are not those seen with isolated tumors in those structures

Bauer There were postmortem findings in the series of acromegalics reported by Cushing and Davidoff (42)

Selye Is hypertension not more common in the acromegalic than in the general population?

Astwood It is The commonest cause of death in the disease is hypertensive cardiovascular disease

Bauer They have cardiomegaly as part of the visceromegaly

Thorn Could we review once more the evidence which suggests that the growth of the adrenal medulla is greater than the increase of other viscera?

Long The question is is the increase in the size of the medulla greater than the increase in body size?

Li Yes only in very high dose normal mice In two instances we found that the medulla was increased in size in short term experiments but not in the hypophysectomized animals

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Dorfman What is the relative sensitivity between the tests *in vitro* and on the alloxan diabetic rat?

Young The alloxan diabetic hypophysectomized rat is about two or three times more sensitive but the amount of insulin activity — I

must not say insulin — in the plasma of the woman patient I mentioned was of the order of a quarter of a unit per millimeter which seems very high One possible explanation might be that there is a large amount of glucagon in the blood of this woman and that is what we are setting out to estimate next

Astwood Drs V W Westermeyer and M S Raben (44) have studied the effect of a pituitary extract on the lowering of the blood sugar A so called corticotropic fraction was given to fed or fasted mice and either at one hour or at three hours the blood sugar was determined

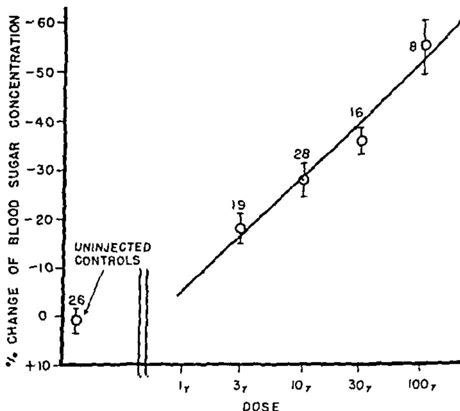


FIGURE 24 Log dose-response curve of the one hour effect of the oxycel purified pituitary extract on the blood sugar concentration of intact unfasted mice Each point is the mean of the number of determinations indicated The ordinate shows the per cent change in blood sugar the uninjected control value is shown in the left lower corner The bars extending from each point represent the standard errors of the means Reprinted by permission from Westermeyer V W and Raben M S Fall in blood sugar from anterior pituitary extract *Endocrinology* 54 173 (1954)

Figure 24 shows the change of blood sugar in one hour The dosage given on the bottom scale shows that with 3 to 100 μg there was a proportionate fall in blood sugar in uninjected animals it did not change during this one hour period Above 100 μg there was no further drop and the animals did not become hypoglycemic to the extent of convulsions

The strange thing about this is that while this fraction caused the drop in blood sugar it was anti insulin at the same time Five units of insulin to the mouse caused convulsions and death but 5 units of insulin given with 100 μg of the extract seemed to have *no effect whatever In other words* the blood sugar fell about the amount that it would have fallen with the pituitary extract alone

White What is the material on the bottom line?

Astwood It is a corticotropin concentrate made with oxycellulose Normal mice were used and it works whether the adrenals are present or not

Li Is the same effect obtained with adrenalectomized mice?

Astwood Yes

Li Is the injection subcutaneous?

Astwood It is either subcutaneous or intraperitoneal

Long It is very reminiscent of the effects of crude pituitary extracts and of some more highly purified growth promoting preparations

Astwood Something of the order of 500 μg of some growth hormone preparations and not others will produce a rather incon stant effect

Ralli By inconstant effect do you mean an inconstant decrease?

Astwood Yes *sometimes it will work and sometimes not* What ever factor it is it is much more greatly concentrated in this case than in the usual growth hormone preparations

Ralli We had a hypophysectomized patient in whom the effect of an Armour growth hormone preparation was studied This patient had an idiosyncrasy about eating and it was difficult to induce her to eat properly The growth hormone was given twice daily for several days and the blood sugars were done in the morn ing and also during the day We found a consistent decrease in blood sugar over a period of three days probably because the patient would not eat I do not know about the purity of the prepara tion used

Astwood We have not been able to separate this type of activity from corticotropin Recently we used modifications of the method of Dixon Moore Stack Dunne and Young (45) which largely

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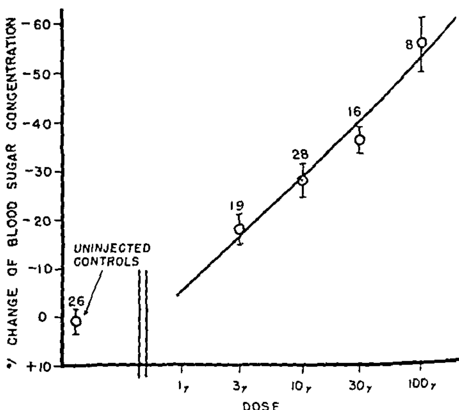


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a larger drop in the treated When maintained with cortisone the effect was a little greater but in neither case was it quite so large as in the intact animal

Long Did cortisone reduce the blood sugar in these animals?

Astwood No Both the controls and pituitary treated animals received cortisone

Li Are you postulating this as a new principle Dr Astwood?

Astwood I do not know I think it is probably just a concentrate of the factor which had been studied previously in growth hormone preparations

Li Do you think that the growth hormone is contaminated with this material?

Astwood That may be or there may be overlapping activities it is difficult to say

White Have you assayed this for growth hormone activity?

Astwood Yes there is no growth hormone activity in hypophysectomized rats

Long What about the fat metabolism factor?

Astwood It is the same extract that has most of the fat mobilizing factor This effect and the fat mobilizing effect whatever it is are very efficiently concentrated in this fraction in Figure 26 These are Dr I N Rosenberg's data (46)

The second curve from the right labeled anterior pituitary is a dose response curve of whole pig pituitary The crude corticotropin constitutes about one eighth the weight of the whole pituitary and the activity is roughly four times as great The oxycel purified corticotropin is perhaps 40 times more active still You will notice that the assay is poor but nonetheless one can estimate that about one half of the active principle is concentrated in this corticotropin fraction which amounts to about 0 2 per cent of the weight of the pituitary powder In other words the factor has been concentrated about 250 fold The problem is whether any of these effects is attributable to corticotropin itself or whether it means that we still have a gross mixture of hormones

Ralli How are you testing for the fat mobilizing effect?

Astwood We use the original method of Campbell (47) measuring the amount of fat in the liver after 3 or 7 hours They have shown that the increased fat in the liver accounted for about 1 5th of the fat that left the depots

Ralli Do you take a sample of the liver before and after administration of the hormone?

separates intermedin but this activity goes along with the corticotropin fraction

Long Does this produce any change in the amino nitrogen of the blood?

Astwood We have not tested that It does produce the glycostatic effect however as Dr John Otto* has shown

Pincus Does it work in alloxan diabetic animals?

Astwood I am not sure about that

Conn Is the adrenalectomized animal no more sensitive to the hypoglycemic effect than the intact one?

Astwood Apparently not The effect is not quite as great in the adrenalectomized animal but it is present It seems to be a bit better if the animal is maintained with cortisone but not significantly so

On the left in Figure 25 the adrenalectomized animals were maintained with saline There was some drop in the controls and

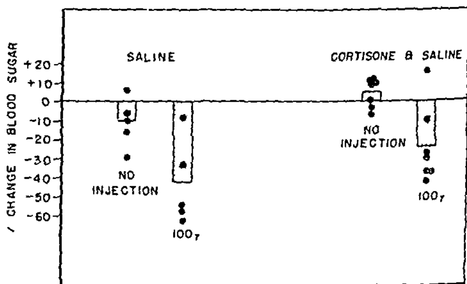


FIGURE 25 Fall in blood sugar in adrenalectomized mice The change in blood sugar at one hour after injection with 100 μg of the oxycel purified material is compared with uninjected controls using unfasted mice adrenalectomized three days earlier The left pair of graphs summarizes an experiment using mice given 1 per cent NaCl in the drinking water the results on the right were obtained with animals receiving 2.5 mg cortisone subcutaneously immediately after operation and saline as well Reprinted by permission from Westermeyer V W and Raben M S Fall in blood sugar from anterior pituitary extract *Endocrinology* 54 *173 (1954)*

*Unpublished data

Astwood No the controls are other animals It is a very variable phenomenon because so many things affect liver fat

White This also happens in the absence of the adrenals?

Astwood Only if the animals are maintained with cortisone

Pincus Did you study adrenal fat?

Astwood No only cholesterol but Dr Charles H Best and J Campbell showed that there was an increase in kidney fat This same extract also causes a drop in respiratory quotient and an increase in oxygen consumption which corresponds very closely with what Drs O Donovan and Collip (48) described years ago as the specific metabolic principle

Bauer What is the change in respiratory quotient (R Q)?

Astwood It goes down at the same time the total oxygen consumption rises and that perhaps would agree with fat mobilization

Long Did you obtain a ketosis?

Astwood Yes All of these effects at one time or another have been attributed to growth hormone but now this fraction contains them all in a much more highly concentrated form

Ralli We have been doing something similar in rats Prior to administering the growth hormone we removed part of the liver in order to study the effect of the growth hormone on the restoration of liver tissue The liver nitrogen lipid and glycogen were determined in each rat before and two weeks after growth hormone therapy At the time of sacrifice the adrenals were assayed for both ascorbic acid and cholesterol Total protein albumin and globulin were determined in the blood An Armour growth hormone preparation was used Control animals were studied at the same time At sacrifice the liver nitrogen was reduced in both the control and growth hormone treated rats The glycogen values in the liver were also reduced The lipid content of the liver was elevated in both groups We felt that the dose of growth hormone should be increased in order to exaggerate any differences that might be present Could Armour s growth hormone be purified by treatment with oxycellulose?

Astwood Dr Rosenberg (46) found Armour s growth hormone to be less than 1/20 as active as this fraction Treatment with large amounts of oxycellulose did not remove any of the activity which leaves open the question of a different substance in growth hormone or perhaps an inherent property of growth hormone

White There is a point which I think arises in such a situation and that is to what extent in an animal with a pituitary the

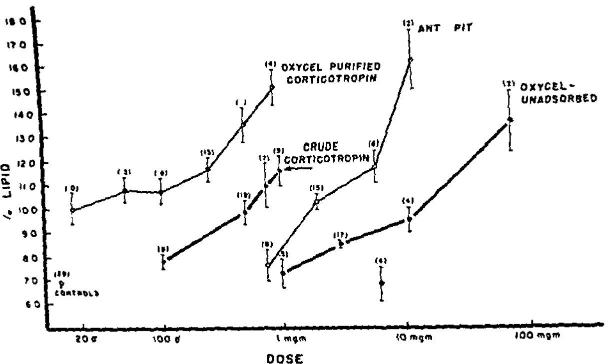


FIGURE 26 Relation between dose of pituitary extract and the per cent of hepatic lipid seven hours after injection in normal mice Comparing the minimal doses required to cause an increase in lipid content from the control value of 69 per cent to a value of 10 per cent it may be noted that oxycel purified corticotropin is about 30 times as active as the crude which in turn is four times as active as the anterior pituitary powder The oxycel unadsorbed fraction from which growth hormone may be obtained is of very low potency Reprinted by permission from Rosenberg I N Adipokinetic activity of oxycel purified corticotropin *Proc Soc Exper Biol & Med* 82 701 (1953)

Young So highly purified ACTH is in effect reducing the blood sugar in the adrenalectomized rat

Astwood Yes

Thorn If the dose is increased does the level of blood glucose change with this material?

Astwood The larger dose does not produce a greater effect it still produces a 60 per cent fall

Ingle Did you say that this preparation also affects the metabolic rate and respiratory quotient?

Astwood Yes

Conn How low does the R Q go?

Astwood To about 0 7 It has been done in mice rats guinea pigs and dogs

Pincus You did not mention what the ACTH activity was Could you state it in International units?

Astwood Most of the work was done with the type of preparation that you have assayed and the assay has been anywhere from 40 to 100 units depending on whose laboratory it was We found it to be about 80 units material which had been further fractionated by the methods developed in Dr Young s laboratory contained about three times that or 240 units

The Armour Laboratories report that oxycellulose corticotropin contains about 40 I U per mg or about half of what we had thought Now comparing material prepared by the Cambridge procedure we obtain about three times that It is impossible by our method of assay to compare with the international standard

Long If we continue injecting the fed animal with a highly purified ACTH preparation will not the blood sugar rise produce the so-called diabetogenic effect?

Astwood Yes with the adrenals present

Long But the initial effect is a fall?

Astwood Yes

Young With the adrenalectomized mouse?

Astwood We have not done that with the adrenalectomized mouse but mice given one injection a day for eight days will still show this drop one hour after an injection

Ralli I wonder whether the continued administration of this material to the normal animal would eventually cause an increase in blood sugar Do you think it is the persistent administration of the hormone that causes the changes in the adrenal gland?

Astwood Presumably it is the adrenal effect

administration of growth hormone may result in some degree of compensatory secretion of an ACTH like factor

Long This preparation is glycostatic in a hypophysectomized rat

White I was speaking here about the fact that Dr Astwood was unable to remove this factor from his growth hormone by oxycel lulose treatment

Li Some years ago we did some studies on hypophysectomized rats in order to examine the effect of growth hormone on liver fat We did obtain an increase in liver fat produced by growth hormone Our results appeared to agree with Dr Astwoods using oxycel lulose or other means we were unable to remove the fat mobilizing property of growth hormone As far as *a* corticotropin our purest ACTH preparation was concerned when it was administered to *adrenalectomized animals it did not cause fat mobilization Only* in mice with intact adrenals did we obtain the fat mobilizing effect with this preparation

Astwood What was the biggest dose you tried?

Li We used 0 25 mg of our preparation assaying approximately 200 U S P units per mg

Astwood What was the time interval?

Li We used an interval of six hours We followed Rosenbergs procedure of extraction (46) including the time as well as the 1936 procedure of Best and Campbell (49) and had the same results each time

Astwood Did you conclude that a separate substance was re sponsible for this?

Li We concluded that ACTH possesses an intrinsic fat mobiliz ing activity as does growth hormone The dose response plotted against the degree of fat mobilization with growth hormone is a very flat curve In the case of ACTH it is a very steep one

Astwood I misunderstood you I thought you said you did not obtain fat mobilization

Li In the absence of the adrenal we obtain no fat mobilization but with the adrenals intact there is fat mobilization with ACTH

Young But you do regard this preparation as one of the best preparations of ACTH that is available? You have had all these other effects

Loeb That is determined on the basis of an ascorbic acid depres sant I assume

Pincus It had a very depressing effect

Astwood I think that is correct

Young What is the difference between the diabetogenic and hyperglycemic factors?

Astwood By diabetogenic factor I mean the factor which produces permanent diabetes in dogs — the Young factor if you will

Young Then hyperglycemic might or might not be the same

Astwood For example is the factor which alleviates the insulin sensitivity of the hypophysectomized dog the same as the factor which produces permanent diabetes when given for a long period of time? It is very difficult to determine

Young What do you include under glycotropic ?

Astwood That, I believe is your own term for the substance which inhibits insulin

Young When you mentioned the factor that relieves the hypophysectomized dog were you thinking of maintaining the blood sugar?

Astwood Yes

Young So the first three factors in Table VII are not clearly differentiated in fact they could all be the same?

Astwood Yes these are poorly differentiated effects

Ralli You were saying that this corticotropic hormone has a hypoglycemic effect?

Astwood Yes

Long And a hyperglycemic effect also?

Astwood I did not say it had a hyperglycemic effect However, I know that if you stimulate the adrenals enough the blood sugar will rise

Loeb Isn t this an ACTH compound that stimulates the adrenal?

Astwood By stimulating the adrenal one can certainly raise the blood sugar but the substances labeled hyperglycemic factor is one that has been referred to in the literature for a long time and is not related to ACTH

Loeb I ask again whether ACTH does not have we will say indirectly by the adrenal a hyperglycemic effect

Astwood Yes I would agree

Long I think what Dr Astwood is saying is that the factors that are present in this extract are effective in the absence of the adrenal but that if the adrenal is present we also obtain all the metabolic effects that are associated with the release of adrenal steroid Do you accept that Dr Astwood?

Astwood Yes

TABLE VII

A Partial List of Metabolic Factors, or Effects, Obtained from Pituitary Extracts

Diabetogenic factor
Hyperglycemic factor
Glycotropic factor
Glycostatic factor
Hexokinase inhibitor
Adipokinin
Specific metabolic principle
Lowering of respiratory quotient
Stimulation of insulin secretion
Stimulation of pancreatic alpha cells

Table VII gives a list of some of the metabolic effects from pituitary extracts that have been described in times past However this is only a partial list The ones that have been associated with the growth hormone in publications are the diabetogenic hyper glycemic glycotropic and glycostatic factors the hexokinase in hibitor the adipokinin the specific metabolic principle a factor lowering the respiratory quotient one stimulating insulin secretion and one the pancreatic alpha cells Those and others, have been ascribed to the growth hormone The ones that we now find to be highly concentrated in the oxycellulose absorbed material are glycotropic and glycostatic factors adipokinin specific metabolic principle with lowering of the respiratory quotient and in addition a lowering of the blood sugar

Ralli Did you say the hexokinase inhibitor was associated with the corticotropic factor?

Astwood No

Loeb You are not including the hyperglycemic factor in ACTH activity?

Astwood No That usually referred to a hyperglycemia which was not mediated by the adrenal

Long But it is present in preparations rich in ACTH

Astwood Yes it would certainly seem reasonable that a single substance could mobilize fat and promote its burning to cause a lowering of the respiratory quotient and an increased oxygen consumption and perhaps spare glucose so it could have an apparent glycostatic effect I do not know about the glycotropic and hypoglycemic effects they are difficult to determine

Bauer You do not think the latter would in any way be concerned with fat metabolism?

Astwood This one substance might be very intimately connected with fat metabolism and do all of those things

Long Have you observed these effects in subjects other than the mouse and the rat? In the human for example?

Astwood The respiratory experiments have been done in rats guinea pigs and dogs

Young Is there any particular reason why the hypoglycemic effect is missing from the list in Table VII?

Astwood It is an old table we did not know about it

Loeb You would put the hypoglycemic factor definitely into the ACTH group but Dr Li says you find the same in growth Is that correct?

Astwood That was shown by Milman and Russell (50) several years ago but the dose was very different it takes many times as much growth hormone to have these effects

Ralli When you remove the pituitary freeze and lyophilize it and then put it on the oxycellulose column how many times do you have to elute it before this substance is obtained? Where does it come off the column?

Astwood The oxycellulose is not used in a column it is used batchwise The minimal amount of oxycellulose is stirred with the crude extract for 24 hours then one elutes with hydrochloric acid yielding the oxycel fraction

Long I would be extremely interested to know if this lowered the nitrogen excretion in rats particularly the crude B alpha extract If it does then we might have an explanation of the hypoglycemic effect when the proportion of proteins in the metabolic mixture is reduced We might very well have a depression of the blood sugar which is merely a reflection of the diversion of the gluconeogenetic material Dr Harrison and I (51) studied this a good many years ago All one has to do is simply fast the animal select one control day and on the second or third day give the preparation one obtains from a 25 to 30 per cent drop in the nitrogen excretion

Loeb You are separating the fact of growth hormone and ACTH on the presence or absence of the adrenal in terms of hyperglycemic activity

Astwood Yes

Thorn Then possibly to be consistent the study ought to be carried out in the absence of the thyroid and in the absence of the islet cells

Bauer But in the absence of the adrenal there would be the glycotropic and glycostatic factors and the adipokinin and the lowering of the blood sugar also Is that correct?

Astwood Yes

Ralli And a decrease in the respiratory quotient

Astwood And the effects described by O Donovan and Collip (48)

Long The lowering of the respiratory quotient is in the absence of the adrenal?

Astwood Yes Dr Rosenberg (46) showed that

Ralli Then this should be labeled corticotropic hormone effects in the absence of the adrenal to make it quite clear

Long As I understand it this preparation has no growth promoting activity therefore you exclude the growth hormone preparation as being responsible for these extra adrenal effects

Bauer There might be some overlapping

Astwood Yes The usual growth hormone preparations though active are very weak when compared with the corticotropic fraction

Long As the preparations become richer and richer in ACTH and yet still contain growth hormone we are forced to use the hypophysectomized and adrenalectomized animal for the assay of growth hormone otherwise the ACTH may completely mask its effect

Astwood That has been done

Ralli Has this hormone factor no adrenal ascorbic acid effect?

Astwood Oh yes it is highly active

Ralli Do you attempt to explain the effects of this hormone in the absence of the adrenal?

Astwood It is unknown whether they are due to one or many substances in that particular extract

Long Would you hazard a guess as to whether we now have to go back to six hormones in the pituitary? There are obviously some close interrelations between a hyperglycemic factor and that which lowers the respiratory quotient

Young Dr Astwood this material is oxycellulose absorbed followed by elution Have you also obtained effects from material that has been put through amberlite IRC-50?

Astwood Only in a preliminary way We have not good quantitative data but from columns such as those used in your laboratory all the data we have so far show that the metabolic effects go along with the corticotropic activity and not with the intermedin

Young Do you find your activity in any one peak in the columns or is it spread over a number of peaks?

Astwood We obtain two main peaks of corticotropic activity and both contain adipokinetic activity We agree with you that there is a large first peak which contains intermedin

Young We originally prepared crude corticotropin by the method which Dr Astwood and his colleagues developed but more recently we have gone back to the Lyons acid acetone method obtaining what is in effect a crude prolactin preparation

We then put it through oxycellulose absorption precisely by the *method that Dr Astwood has developed and ran the extracts* which assayed about 30 I U I believe you obtained 40 I U

Then we ran this through a column of amberlite IRC 50 by the method we published (52) and obtained four peaks of activity In Figure 27 is the main peak which we call A_1 then A A_3 and A_4 We find that the material in these peaks runs something like 50 I U per mg We have done a little better than that but we cannot get up to the 100 at least not consistently The original glands have been extracted by a number of different methods We have not yet investigated these peaks for different physiological activities but have been anxious to know whether each one would behave as a single substance on rechromatography or when other treatments were used

When peak A_1 is again put through a column of amberlite IRC 50 it comes out at the same place as it should do if it were behaving as a single substance We have also subjected a material to chromatography by a system Hess and Carpenter (53) have used and again find it behaving as a single substance We have done some paper electrophoresis and also can obtain no evidence of separation of this peak into more than one substance likewise the peak A will *consistently rechromatograph* and also peak A

We have observed more recently that if one treats peak A_1 with ammonia or with any alkaline medium around pH 11 or 12 it is converted into material that is indistinguishable in our hands from peak A so that *some change has occurred in the alkaline medium*

This occurred also in the adrenalectomized animal given the crude extract

Thorn Did these changes occur in the nonprotein R Q value?

Long One has to do the urea nitrogen

Thorn We have to be very careful in studying the lowering of respiratory quotient in an adrenalectomized animal It is important to know the exact time at which it is measured If an adrenalectomized animal responds as a patient with Addison s disease then we know that these individuals will maintain a relatively high R Q for a short period of fasting but that a break may suddenly occur *At this point the respiratory quotient falls to quite low values* If we should happen to give hormone to the fasting adrenalectomized animal at or about the time the spontaneous fall in R Q might have occurred we would not be certain of whether the material administered was responsible for the fall in R Q or whether this was a natural occurrence

Long That is why I asked whether a larger animal would be used I do not think the fasted dog would show these rapid fluctuations They are fed anyway

Astwood Most of the R Q s were done in the fed animal and some with continuous infusion of glucose We have arranged a mechanical device for measuring oxygen consumption and CO production and can obtain an almost continuous measurement of these two The animal may be arranged in a chamber with a cannula placed intravenously subcutaneously or intraperitoneally and after obtaining a base line of oxygen consumption and CO production injections may be carried out without disturbance Thus the time course of these effects may be easily followed

Long I have no doubt it all that these effects can be produced by pituitary extracts they have all been well substantiated The interesting thing is that we now have this activity in a much more highly purified state

You said 3 μg of the oxycellulose preparation would produce a measurable effect on the blood glucose How much of that is ACTH 50 per cent? Five per cent? It is an extraordinarily active hormone

Astwood I do not know I failed to mention that if one grinds up beef pituitary glands all the ACTH virtually disappears as Dr Sayers has also shown The Parke Davis and Company thyrotropic hormone preparation is made from fresh beef and sheep glands and next to this preparation it is the most potent so far as adipokinetic activity is concerned it has no corticotropic activity

which is responsible for a shift in position on the columns in keeping with a change in iso electric point However one cannot say for certain what the iso electric points are

I asked about the activity of the various types of biological response that Dr Astwood has been dealing with in these various peaks because I wondered whether one had a spread of activity in other tests such as we have found with ACTH We certainly did not obtain one main peak as Hays and White (54) and I think some other workers found Dr Astwood you detected two main fractions but did you not find anything corresponding to our A_3 and A_4?

Astwood In general we obtained just two corticotropic peaks We have noticed without deliberately trying to treat with alkali that on rechromatographing these peaks the slower peak seems to turn into the faster one

Young How did you desalt your peaks before the rechromatographing?

Astwood Either by precipitating with picric acid and regenerating or else by using coarse Dowex 50 resin in the acid cycle

Young We were I think misled for a time by using a desalting method which involves ammonia and ammonia treatment under the conditions we were using causes the partial transformation of peak A_1 to A but if one uses a method for desalting which we now have developed involving shaking up with phenol and precipitation of the active material from the phenol by addition of ether one can obtain consistent A_1 activity out of the A_1 peak without any transformation to A

Li What solvent do you use for this resolution?

Astwood Bicarbonate buffer 0 1 Molar pH 9

Li And this is a sort of elution analysis or pH change?

Astwood We have followed the method used in Dr Young's laboratory

Young Your pH is 5 we have used pH 6 6 or 6 8 and we seem to obtain a fairly good differentiation One wonders if there is one ACTH in the gland which is being divided into a number of fractions here or whether all of these things are pre existing The impression one gets from publications from the Armour Laboratories is that there is just one corticotropin A

Astwood I still rather like the idea based upon physiological considerations that there are separate substances giving rise to these several effects Apparently the intact animal has a way of regulating the adrenal cortex separately from the regulation of these

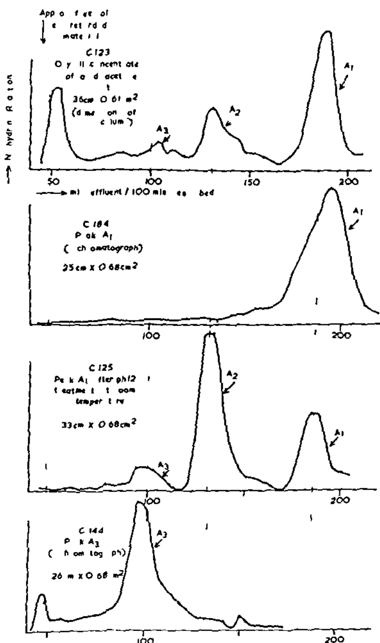


FIGURE 27 Chromatography of an oxycellulose concentrate of an acid acetone extract of pig pituitary tissue on amberlite XE 64 at pH 6 70

Li Yes The amino acid content accounts for over 95 per cent of the peptide nitrogen

Astwood Would you hazard a guess as to the size of the material after pepsin?

Li It would be difficult to say but I think it should be smaller than 4 500

For the peptic digestion of the unhydrolyzed product we use an entirely different system The peak in the middle we call B_1 and B_2 The B_1 and B_2 are both active biologically but B_2 can be converted to B_1 very easily and we obtain only a single B_1 The D fraction sodium hydroxide treated has a pH of about 12 and peak A comes out about the same

Ingle I might tell a little story about a biological effect which we once thought was an inherent property of ACTH We had developed the work test for the bioassay of adrenocortical hormones Whereas a normal rat can work very well for periods of several days the adrenalectomized rat goes into collapse within a few hours time We found that if we gave a sufficient amount of adrenocortical extract to the adrenalectomized rat by continuous intravenous injection it would work as well as a normal rat The untreated hypophysectomized rat also works very poorly but its work performance is improved by treatment with either adrenocortical extract or by corticotropin Using commercial preparations of corticotropin given by continuous intravenous injection we were able to make the hypophysectomized rat work just as well as the normal rat

We assumed that cortical extract should also restore the performance of the hypophysectomized animal to normal but instead the recovery was only from 60 to 65 per cent of normal value Corticotropin was doing something that cortical extract would not do We found this to be true of three commercial preparations of corticotropin and of several other experimental preparations obtained from Drs Astwood and Li of this conference and from Mr *John Nelson in the Upjohn* Laboratories There was a *marked* discrepancy between the apparent effect of corticotropin in the work test and in the Sayers test and we suspected that these activities were not parallel

The studies were then extended to the adrenalectomized hypophysectomized animal Again treating the animal with adrenocortical extract partially restored work performance When we added corticotropin work performance was restored to normal even though the adrenals were not present During the past year we have

other phenomena so that if these other metabolic effects have physiological significance one would prefer to attribute them to some substance other than the one affecting the adrenal cortex

Li We have recently been able to obtain an adrenocorticotropic hormone from an unhydrolyzed ACTH concentrate of sheep pituitary glands (55) which behaves as a pure peptide in various physicochemical and biological tests and is different chemically from the preparations reported by the Armour and Merck groups There are three steps in our procedure First from a solution of Fraction E (56) in 50 per cent dioxane at pH 9 3 9 4 the inactive material is precipitated out The supernatant is then submitted to zone electrophoresis on starch The active fraction obtained thereby is then chromatographed on amberlite XE 97 resin which is similar to IRC 50 From this column is obtained an active fraction which is further purified by being submitted to countercurrent distribution in a 2 butanol per 0 1 per cent trichloroacetic acid system Those tubes whose contents follow the theoretical distribution curve for a partition coefficient of 0 41 yield a product which behaves as a single substance as judged by various additional criteria of purity such as (*a*) zone electrophoresis on starch in various buffers at different pHs (*b*) elution analysis on XE 97 resin columns (*c*) end group analysis (*d*) chemical composition and (*e*) biological assay Since this product possesses physicochemical characteristics which differ from the unhydrolyzed preparation corticotropin A of Armour we propose to designate it as *α corticotropin* The molar ratio of the amino-acids in α corticotropin is as follows alanine 3 arginine 3 aspartic acid 2 glutamic acid 5 glycine 3 histidine 1 leucine 1 lysine 4 methionine 1 phenylalanine 3 proline 4 serine 3 tryptophan 1 tyrosine 2 and valine 3 The minimum molecular weight of α corticotropin is approximately 4 500 The iso electric point has been estimated to be located at pH 7 0

Long What is the biological activity?

Li Between 150 and 200 We are not really sure one day we get 150 and another day 200

Astwood To which of Dr Youngs peaks would your peak correspond?

Li It is difficult to say because I do not know Dr Youngs solvent system

Astwood But the order of emergence would be the same

Mason When you add up all those amino acids do they add up to 100 per cent?

Ingle The volume is 20 ml of solution to a 200 gm rat in a 24 hour period

Astwood Is there anything to be observed in these rats that would suggest how this effect is being brought about?

Ingle No We have not attempted to carry out any of the metabolic studies on these animals When Dr Marthe Vogt of the University of Edinburgh visited my laboratory she called my attention to a peculiar difference between the animals that received this principle and those that did not The rat if it is anesthetized over a long period of time such as 24 hours or so is likely to extrude a pigment from the eyes which is related chemically to hemoglobin it does not represent blood itself She noted that the animals receiving this pituitary fraction had much more pigment about their eyes and that is true I have no idea why

Astwood That is of interest because I believe the secretion of that material in the eye is brought about by cholinergic drugs We have isolated one pure substance from the corticotropic concentrate We have had it in crystalline form for some three years as a picrate and it was only recently that we accumulated enough to find out what it is It turned out to be spermine much to our disappointment because spermine crystals were described by Lewenhoeck (57) nearly 300 years ago It was not a new discovery

Long And widely distributed in the body

Astwood Yes

Bauer That is right Dr Rene Dubos of the Rockefeller Institute found that to be true

Selye We use a procedure in which rats are forcefully immobilized in order to produce stress This invariably causes chromodacryorrhea which is I think the correct designation for this condition We have also seen it in forcefully immobilized adrenalectomized rats

Long We certainly do not seem to have solved the question that was posed to us by Professor Young as to whether ACTH is a single substance or a mixture of hormones I suppose the only thing we can say is that certain preparations of ACTH are most certainly a mixture of hormones Whether there has yet been prepared a substance that represents the real tropic hormones of the adrenal cortex is undecided Do you agree Dr Astwood?

Astwood Yes

Long How about you Dr Li do you think you have free ACTH now?

used Merck's corticotropin B Armour's corticotropin A and the pure preparation of corticotropin that Dr Li has just described to you These purified preparations do not have this extra adrenal effect unless relatively large doses are administered Dr Li has a fraction which is active in as little as 1 μg per rat per day This fraction does not have any significant amount of corticotropic activity

We have tested the other purified pituitary hormones They do not have this effect nor does intermedin So we appear to be dealing with a principle that has not been identified It is possible that we are dealing with a mixture of known principles but this does not seem probable It is possible that we are dealing with a principle which somebody else has measured by another criterion for example one of the criteria which Dr Astwood has been discussing Whatever it is our data do not seem to be explicable in terms of the principle which causes depletion of adrenal ascorbic acid

Long It is an extra adrenal factor

Ingle Yes

Li This factor that Dr Ingle mentioned also has had no effect on adrenal size

Astwood Where did it emerge on the column in the fractionation that you just described?

Li It is the first peak derived from the XE 97 column using the pepsin digest of purified ACTH

Astwood With the intermedin?

Li Yes that fraction is quite a mixture

Mason How do you know it is not intermedin?

Li I think Dr Ingle tested not only our own highly purified intermedin but also another preparation

Ingle Yes one which we obtained from Armour

Mason That intermedin I believe has a little ACTH activity in it

Ingle Yes but very little It is a fraction of a unit per milligram One other point should be made clear In order to demonstrate this effect one must first treat the animal with adrenocortical extract or with one of the 11 oxysteroids An optimum amount of adrenal hormone gives partial replacement By adding this factor there is full replacement

Bauer Do they receive glucose all the time?

Ingle They do not receive glucose There is 0 9 per cent salt in the solution

Bauer And that amount would be how much salt per day per rat?

Long Because ACTH has often been given by continuous drip hypoglycemia might well have been reported if it were a fairly frequent occurrence Have you observed it Dr Conn?

Conn I have never seen any hypoglycemic phase preceding the hyperglycemic phase with ordinary ACTH in man Some of the things that Dr Astwood has mentioned as characteristic of this potent fraction reminds one of that peculiar disease that Dr R D Lawrence has described (59) He calls this lipo atrophic diabetes It is characterized by tremendous oxygen consumption without thyrotoxicosis hyperglycemia and hyperlipemia with great insulin resistance no ketosis almost complete loss of subcutaneous and depot fat and gradual enlargement of the liver with ultimate hepatic cirrhosis Whatever this disease is Lawrence described two such cases Removal of the thyroid lowered the oxygen consump tion only slightly Without any thyroid there was still a very high oxygen consumption

Bauer We have seen a case very much like that It turned out to be hemochromatosis with involvement of the myocardium The autopsy findings explained a lot of things observed during life I wish the two reported cases had been autopsied then we would know for sure what they had

Conn One was autopsied and revealed fatty infiltration of the liver and cirrhosis The fat depots as well as the subcutaneous fat were all gone

Bauer There is another situation which should be considered and that is the end stage of dermatomyositis It can give a clinical picture of the type you described including a fatty liver I do not think we know what disease Lawrence reported

Young In Dr Lawrence s case the basic metabolic rate went up to 130 at one stage I think it is one of the highest that ever has been described

Bauer He also mentioned a couple of cases of dermatomyositis

Conn Have you speculated on where that blood sugar has gone? Because the R Q is down it can be oxidized but cannot be con verted to fat It could go into the liver as glycogen Was there any increase in liver glycogen?

Astwood This is all quite recent Dr V W Westermeyer* has experiments going at the moment to try to answer these questions but he does not know as yet

Long As I suggested before it may have some effect on the protein metabolism

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*Personal communication

Li As a matter of fact we have had a corticotropin for about a year and we have been trying to prove that it is pure If it is then I believe that we shall have solved this problem

Long Does this have a hypoglycemic action?

Li We have not yet tried it However we feel that this material contains fat mobilizing activity A pure hormone could have more than one effect both cortisone and oxytocin have Insulin also has more than one biological property

Loeb In the case of insulin I think one has to say whether one is talking about the primary direct effect or a secondary series of changes

Li The fat mobilizing fraction has to be tested in the presence of the adrenal No fat mobilizing effect occurs in the absence of the adrenals

Loeb All I am trying to say is that it is possible the fat mobilization is secondary to a change which is taking place we will say in the utilization of glucose?

Li Yes

Ingle If one gives a sufficient amount of cortisone fat can be mobilized in the liver Dr Louis Levin (58) has shown that one cannot account for all of the fat mobilizing effect of pituitary extracts in terms of adrenal mediation

Long Dr Kendall didn t you point out some time ago that compounds A and B had more fat mobilizing activity than E and F?

Kendall I think that may be because the animals that had E did not live long A and B had very little effect on the animal except to suppress growth whereas E produced loss of weight and lesions in the muscles and killed a large number of animals But with A and B there was very little else that happened they ceased to grow and fat was deposited where protein should have been

Thorn Has anyone ever observed any improvement in a patient with Addison s disease given ACTH or any striking metabolic change other than the retention of water?

Loeb We tried it in a desultory way but nothing happened

Long I should like to ask if anyone has observed a hypoglycemic effect in man given an ACTH preparation whether intravenously or any other way

Astwood Dr M S Raben* did one experiment in an untreated Addisonian giving I think 5 mg of this preparation intravenously and there was no significant change in the blood sugar This needs to be studied further however

*Personal communication

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Conn Suppose you assume that you completely stopped the glyconeogenesis then the subsequent disappearance of glucose from the blood must indicate that it has reappeared somewhere as glycogen or has been oxidized That would seem to be the only explanation

Long If you stop it coming in that is the natural way

Thorn Did you observe hyperventilation in these animals during the initial phase of the infusion? This of course might make a marked difference in the apparent R Q

Astwood With large doses in rats and mice, one can see an increase in actual physical activity, and the animals suffer from a high environmental temperature I think it is true though that the increase in metabolism in association with a lowering of R Q can occur without any visible change in physical activity including respiration It has with large doses an analeptic effect When one does the experiment under barbiturate anesthesia the animals tend to wake up

Young I should just like to comment on the question of multiple effects of peptide hormones that have been described Perhaps now that synthetic peptide hormones are becoming available there is a chance to test whether there is intrinsic multiple activity

Long I know of no effects attributable to the posterior lobe hormones in such small quantities Quite large amounts must be given if there is to be any effect on the blood sugar

Young Not with regard to resistance to the intravenous action of the insulin In 1941 an experiment was made (*60*) in which significant effects were obtained with I think one or two units or less of oxytocin That was in response to intravenously administered insulin In those experiments animals differed one from the other Some showed a response and others did not The animal that showed the response would do so consistently

Astwood Some of the effects described by Dr Collip in 1941 (61), were obtained with posterior lobe extracts as well as with anterior if I am not mistaken

Young Dr Collip did obtain metabolic effects with intermedin

Astwood He later showed that the specific metabolic principle was separable from intermedin

Ingle May I ask whether it is clear that the antidiuretic principle of posterior lobe is identical with the pressor principle?

Young There is no evidence to the contrary yet that I know of

Li The pure and synthetic oxytocin of du Vigneaud has pressor antidiuretic and about 20 *per cent* oxytocic activity Obviously a pure substance can have more than one biological effect

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INDEX

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